# A NOVEL ROLE FOR THE ADAPTOR PROTEIN AND ARF GTPASE-ACTIVATING PROTEIN CAT-1/GIT-1 IN CELLULAR TRANSFORMATION

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# A NOVEL ROLE FOR THE ADAPTOR PROTEIN AND ARF GTPASE-ACTIVATING PROTEIN CAT-1/GIT-1 IN CELLULAR TRANSFORMATION

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Cornell University, 2012

Cat-1/Git-1 is a multi-functional protein that acts as a GAP (GTPase-activating protein) for Arf GTPases, as well as serves as a scaffold for a number of different signaling proteins. Cat-1 is best known for its role in regulating cell shape and promoting cell migration. However, whether Cat-1 might also contribute to cellular transformation is currently unknown. Here we show that ~95% of cervical tumor samples examined over-express Cat-1, suggesting that the up-regulation of Cat-1 expression is a frequent occurrence in this type of cancer. We further demonstrate that knocking-down Cat-1 from NIH3T3 fibroblasts expressing an activated form of Cdc42 (Cdc42 F28L), or from the human cervical carcinoma (HeLa) cell line, inhibits the ability of these cells to form colonies in soft agar, an *in vitro* measure of tumorgenicity. The requirement for Cat-1 in the anchorage-independent growth of HeLa cells is dependent on its ability to bind paxillin, while its Arf-GAP activity had a negative effect. Collectively, these results suggested that Cat may be acting as an effector for activated Arf GTPases in promoting cellular transformation, and the binding of Cat to paxillin is a critical step in mediating the effector function. In support of this idea, the co-expression of Cat-1 and an activated form of Arf6 in fibroblasts was sufficient to induce transformation in normal NIH3T3 fibroblasts. These findings highlight novel roles for Cat-1 and its interactions with the Arf GTPases and paxillin in oncogenic transformation.

We then go on to show that paxillin, and Hic-5, a close homolog of paxillin that also binds to Cat, act to negatively regulate the anchorage-independent growth of HeLa cervical carcinoma cell line as read-out by soft agar assay. Also, we show that binding of Cat to paxillin, but not Hic-5, is the critical function of Cat that is important for mediating the role of Cat in promoting anchorage-independent growth. These findings suggested that Cat promotes cellular transformation by binding to paxillin to block paxillin from negatively regulating cellular transformation.

# BIOGRAPHICAL SKETCH

Sungsoo was born in Madison WI to a poor graduate student majoring in Economics and his wife, and this would not have been possible without the great help from the Wisconsin government in the form of generous food stamps. Ever since he was four, he lived in Seoul with his grandparents. Although he was always curious about nature, it was probably the last words of his grandfather who miraculously came back to his senses after long departure from his acquaintances due to the effects of the Alzheimer's, that solidified the idea of pursuing a career in academia; " Sungsoo, you MUST study hard." Thanks to such hauntingly inspiring words, he was able to study in the top private university in Korea, but was clueless about what he wanted to study. He just knew that he wanted to earn more than his dad. Having heard the rumor that the field of the Interface between Chemistry and Biology was the future of humanity he took the plane ride to Ithaca, to be part of the Chemistry and Chemical Biology program at Cornell. Turns out there were like 2 professors out of almost 30 studying Chemical Biology, including Rick Cerione. Despite the subtle nudge from Rick to join the other lab, Sungsoo failed to pick up on the signal, and fortunately was able to study molecular/cellular biology in the Cerione laboratory. Although Sungsoo didn't know the difference between Chemical Biology, Biochemistry and Molecular Cellular biology at the start of his PhD, now he is certain he made the right choice. Also he is certain that he will be making even less money than his dad but that's all fine. It's fine because he has a great conviction that with the experience in this lab, he is bound to make great findings in the near future.

In loving memory of Jae-Ryong Michael Yoo

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I would like to thank my parents for their endless support.

Rick, for providing everything necessary for me to learn and enjoy science.

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The committee, for letting me out… and enjoy more science.

And all the people I have interacted with to get here, including the people I might have hurt.

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## Introduction

Normal cells require tight regulation over mitogenic signaling to achieve the appropriate growth and proliferation of cells. Insufficient signaling in this context prevents cells from achieving the appropriate rate of cell growth. However, excessive mitogenic signaling can potentially result in a cancerous growth. Thus, signaling needs to be tightly controlled and it is critical to understand the mechanisms underlying this regulation in order to fully appreciate how disease states progress (i.e., cancer) and to suggest novel points of intervention for cancer therapies. The purpose of this thesis is to determine the role of Cool Associated Tyrosine phosphorylated - 1 (Cat-1) / G protein-coupled receptor kinase interacting ArfGAP- 1 (Git-1) in promoting the aberrant growth of transformed cells and human cancer cells, as well as to gain insight into the molecular mechanisms through which Cat-1 mediates its effects.

### Initiation of Signal Transductions

It is well established that normal cells require the regulation of mitogenic growth signals in order to proliferate (1). Among the best-known examples of such signals are diffusible growth factors, which initiate their effects when they bind to their corresponding transmembrane receptors that reside on the plasma membranes of the receiving cells. Nearly all cell types express several different types of receptors, poising them to respond to a variety of cues that a cell may receive from its surroundings. One of

the best studied group of receptors is the superfamily of receptor tyrosine kinases that include the epidermal growth factor (EGF) receptor, the insulin-like (IL) growth factor receptor, and the platelet-derived growth factor (PDGF) receptor.

One of the earliest identified and best-characterized growth hormones is the epidermal growth factor (EGF). When Rita Levi-Montalcini observed that the transplantation of mouse tumors into chick embryos spurred the growth of nerve cells near the tumor and induced their differentiation, it was realized that there must be some factor coming from the tumor cells that elicited this biological response from the chick nerve cells (2). Using newborn mice as a model system to follow-up these observations and identify the factors that were prompting cell growth and precocious differentiation, Stanley Cohen purified the polypeptide that was responsible for this biological activity, which was later named Epidermal Growth Factor (EGF) (3). He also participated in the purification of the factor that was responsible for Rita Levi's observation, which was named Nerve Growth Factor (NGF). Since their early findings, numerous studies have shown that the signaling events initiated by EGF are intimately linked to normal cell growth and proliferation. Moreover, de-regulation of EGF-mediated signaling events, either by overproduction of EGF or by over-expression of the EGF receptor (EGFR), has been linked to the development of human cancer, including those derived from the brain, lung, colon, and breast (4).

The initial binding of EGF to the EGFR, and the subsequent series of conformational changes that the EGFR undergoes to become activated is a good example of the high

degree of regulation to which mitogenic signaling pathways are subjected. The EGFR contains an extracullular domain which is connected to the cytoplasmic tyrosine kinase domain by a single transmembrane region (5) (Figure 1.1). EGF receptor signaling is initiated when EGF binds to the extracellular domain of the receptor, which is composed of the EGF-binding domains and dimerization motifs (6,7). The binding of EGF to the EGF-binding domains on the receptor induces the exposure of the dimerization motifs, allowing monomeric EGFRs to come together to form dimers. Juxtapositioning of the cytoplasmic domains of the EGFRs via the dimerization of their extracellular domains is thought to induce the receptor's kinase activity. Interestingly, the kinase domains of the EGFR come together in an asymmetric fashion (8),(9) (Figure 1.2). The kinase domain is composed of two lobes, an N-terminal lobe and a C-terminal lobe, and the asymmetric positioning of the C-terminal lobe of one EGFR monomer with the N-terminal lobe of the other EGFR monomer enables the C-terminal lobe to act like a cyclin in the cyclin/Cdk complex, and pull the activation loop away from the active site of the N-terminal lobe on the neighboring receptor, rendering the active site accesible for the binding of ATP. This results in the transphosphorylation of several tyrosine residues found within the C-terminal tails of the adjoining monomers making up the EGFR dimer.

Through EGF-induced dimerization and trans-autophosphorylation of the EGFR, the receptor is now able to activate a myriad of signaling pathways. It does so by recruiting signaling proteins that contain Src Homology 2 (SH2) or Phophotyrosine Binding (PTB) domains to specific tyrosine residues that are phosphorylated on the EGFR. A classical example of this involves Grb2, a SH2-domain containing protein that engages the

**Figure 1.1 A schematic representation of EGF-triggered dimerization of the extra-cellular domains of the EGF-receptor.** EGF binds to the EGF-binding domain in the extra-cellular domain of EGFR, which induces conformational changes that expose the dimerization arms of the EGFR. The dimerization arms interact with that of another monomeric EGFR, bringing the two receptors together. (Adapted from (5))

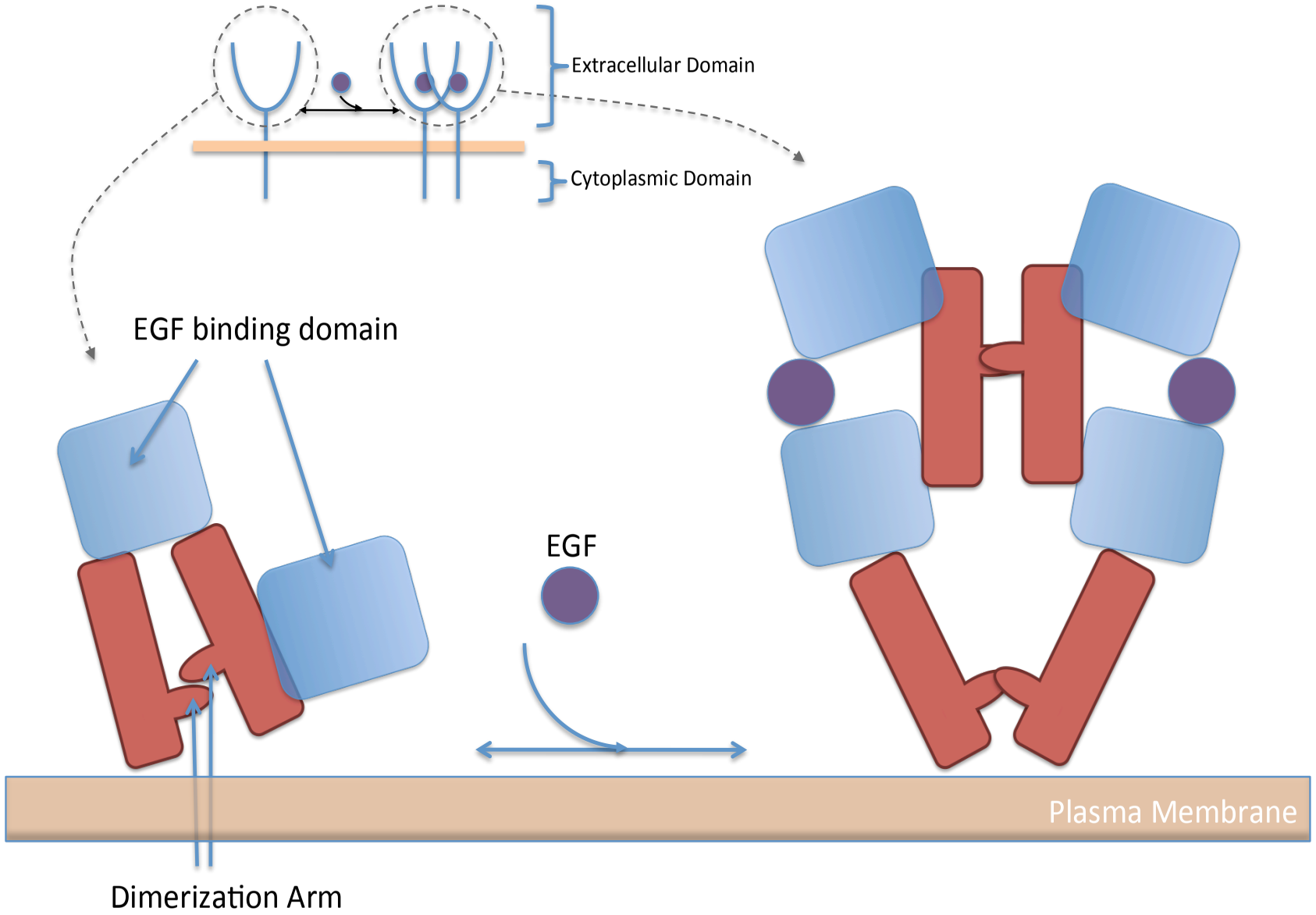
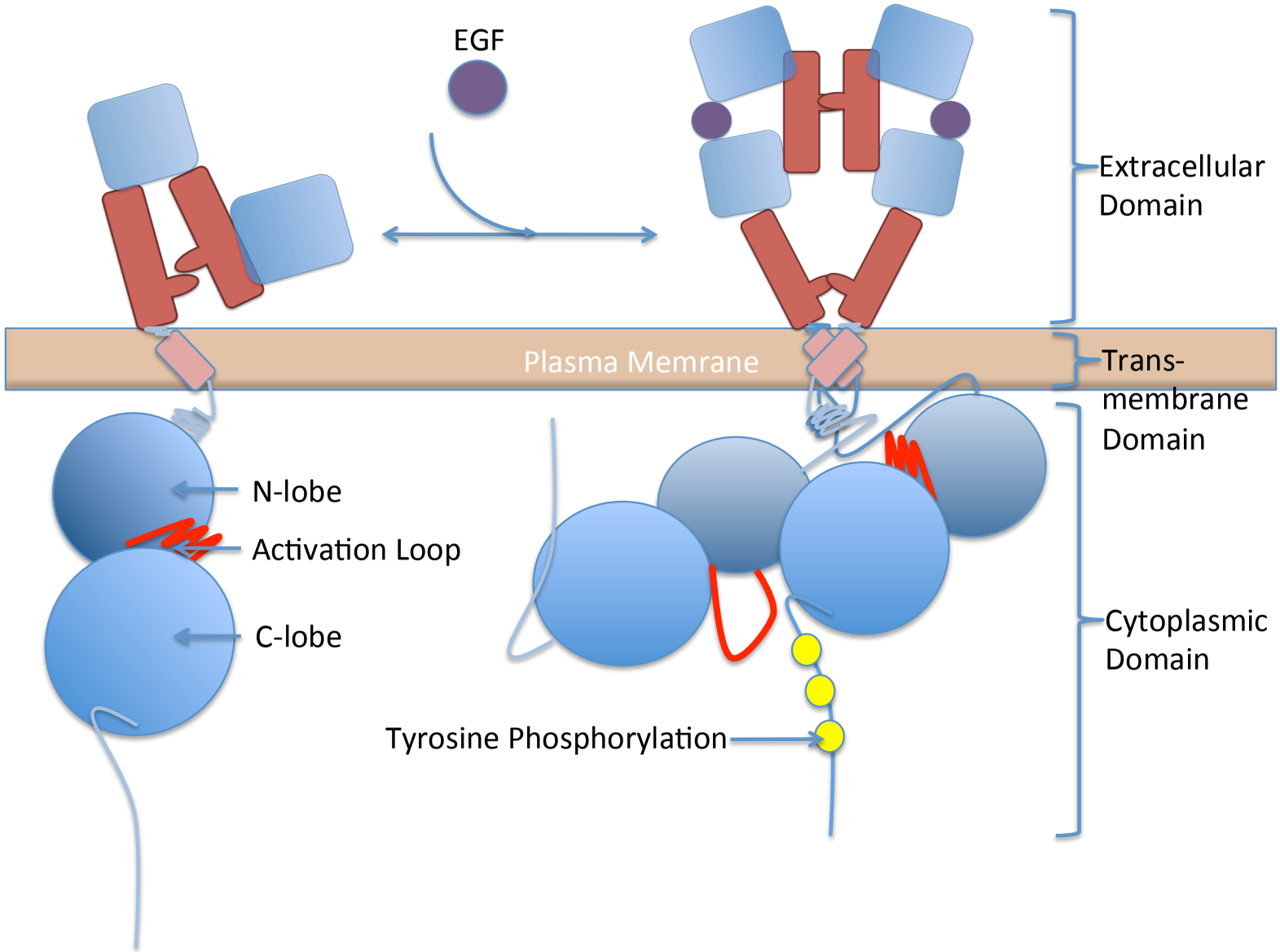

Extracellular Domain
Cytoplasmic Domain
EGF binding domain
EGF
Plasma Membrane
Dimerization Arm

**Figure 1.2 Dimerization of the extracellular domains of the EGFR leads to the activation or trans-phosphorylation of the EGFR via asymmetric interaction of the cytoplasmic domains of the EGFR.** The dimerization of the extra-cellular domains of the EGFR triggered by EGF binding is conveyed to the cytoplasmic domains bringing them in close proximity to one another. The crystal structures indicate that the cytoplasmic domains of the EGFR interact in an asymmetric fashion; the C-lobe of one monomer interacts with the activation loop of located within the N-lobe of another monomer, similar to the cyclin (C-lobe) and cyclin-dependent kinase CDK (N-lobe) activation mechanism (Adapted from (6)).


EGF
Extracellular Domain
Plasma Memrane
Trans-membrane Domain
N-lobe
Activation Loop
C-lobe
Cytoplasmic Domain
Tyrosine Phosphorylation

phosphorylated EGFR, resulting in the activation of the Ras-Erk mitogenic pathway in cells. In addition to activating the Ras-Erk signaling cascade, the EGFR is also able to induce the activation of several other signaling proteins that work together in a coordinated fashion to regulate cell cycle progression, as well as cause changes in the expression pattern of specific genes. These signaling events downstream of the receptor must be tightly regulated in order to maintain a normal growth rate in cells. An important feature of this regulation is the role played by members of the Ras superfamily of GTPases. These proteins undergo a GTP-binding/GTP hydrolysis cycle that allows them to act as molecular switches. When in the GTP-bound state, they engage and activate their signaling partners ("effectors"), whereas GTP hydrolysis serves to switch off the GTPase as thereby terminate its downstream signals.

**Ras superfamily of GTPases**

There are over 60 members of the Ras superfamily of GTPases. They are commonly divided into five subfamilies; Ras, Rho, Rab, Arf, and Ran (Figure 1.3), based on the sequence homologies. These proteins have similar molecular weights that typically range from 21 kDa to 25 kDa, and they are all capable of binding and hydrolyzing GTP, although their intrinsic GTP hydrolytic activity is typically low. Importantly, the activation of these proteins is dependent on whether they are bound to GTP or GDP, hence their classification as 'molecular switches' (Figure 1.4). Despite the high degree of sequence homology that exists between the members of the Ras superfamily, each member has been shown to have distinct functions within the cell. For example, a well-characterized and specific role for the Rho subfamily which includes Cdc42, Rac, and

**Figure 1.3 The Ras superfamily of small GTPases.** The Ras-superfamily is made-up of the Arf, Rab, Ras, Ran, and Rho subfamilies of small GTPases. Each small GTPase functions as a molecular 'switch' that regulates specific signaling pathways within cellRho is their ability to impact cell morphology. On the other hand, the Arf and Rab families have been implicated in the trafficking of intracellular vesicles. A diagram highlighting the members of the Ras superfamily and some of the roles that they play in cells is shown in Figure 1.3.

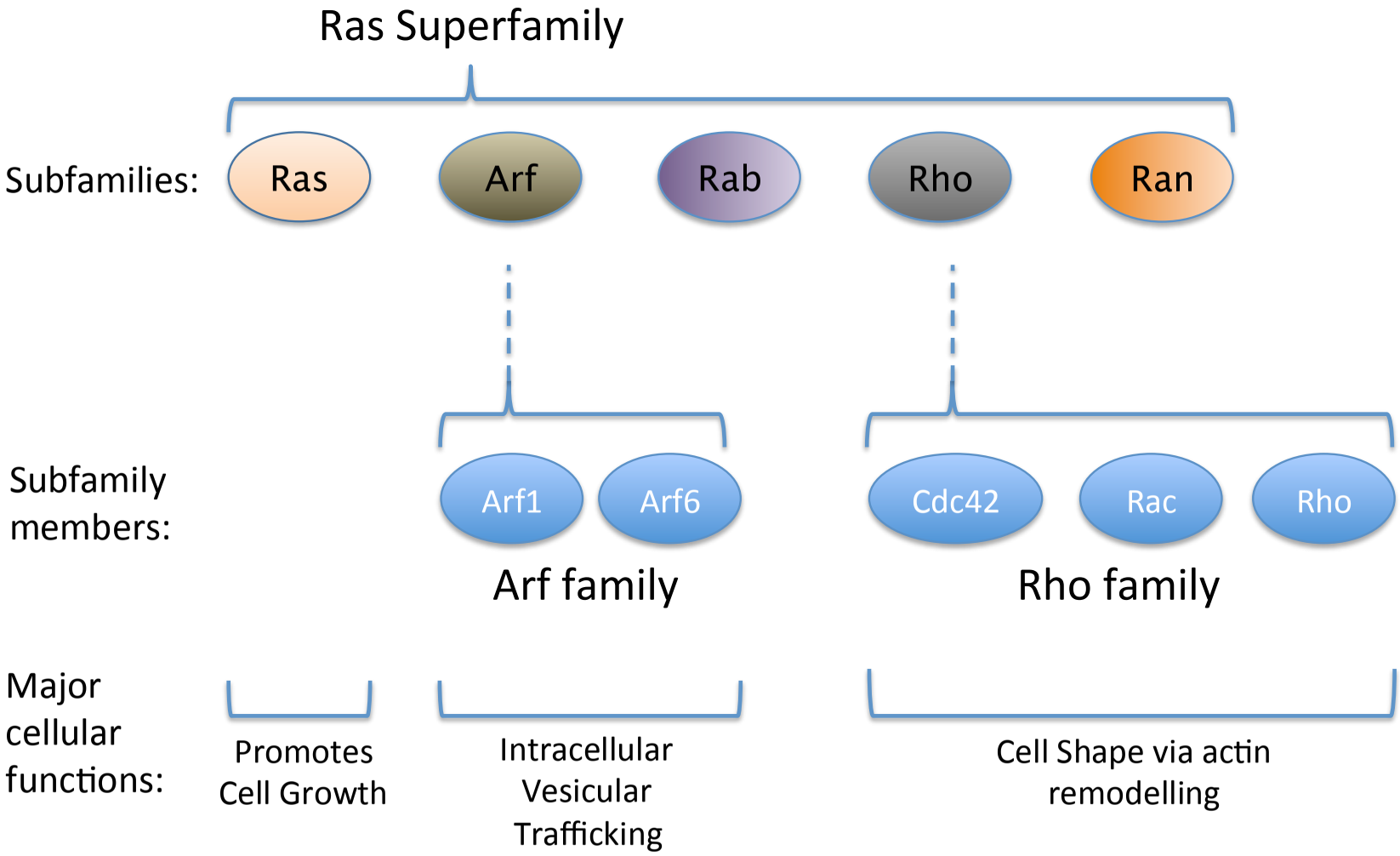
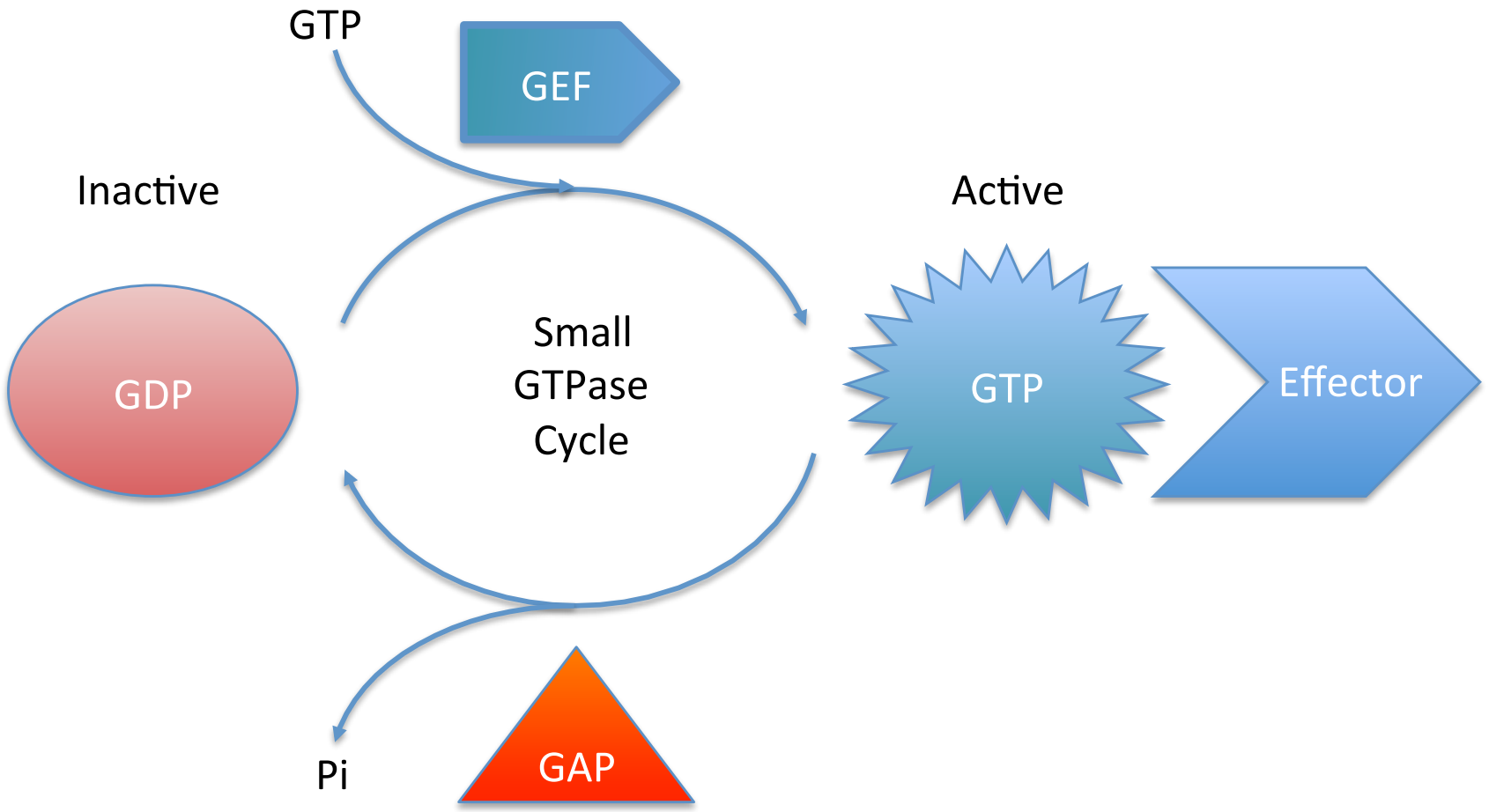

Ras Superfamily
Subfamilies:
Ras
Arf
Rab
Rho
Ran
Subfamily members:
Arf1
Arf6
Cdc42
Rac
Rho
Arf family
Rho family
Major cellular functions:
Promotes Cell Growth
Intracellular Vesicular Trafficking
Cell Shape via actin remodelling

**Figure 1.4 GTP/GTP-hydrolysis cycle of the small GTPases.** All small GTPases are turned 'on' or able to bind and activate downstream effectors only when they are bound to GTP. The binding of GTP is catalyzed by a family of proteins called Guanine nucleotide Exchange Factors (GEFs). On the other hand, GTPase Activating Proteins (GAPs) regulate the de-activation of the small GTPases, by catalyzing the hydrolysis of the bound GTP to GDP. Importantly, many small GTPases require cycling between their GTP/GTP-hydrolysis states to fully achieve their cellular functions.


GTP
GEF
Inactive
Active
GDP
Small
GTPase
Cycle
GTP
Effector
Pi
GAP

Rho is their ability to impact cell morphology. On the other hand, the Arf and Rab families have been implicated in the trafficking of intracellular vesicles. A diagram highlighting the members of the Ras superfamily and some of the roles that they play in cells is shown in Figure 1.3.

Ras was the first protein to be identified in the Ras superfamily and it is best known for its effects on cell growth. It was identified in 1979 as a transforming element of the Harvey and Kristen rat sarcoma virus by Edward Scolnick (10). In 1982, Geoffrey M. Cooper at Harvard (11), Mariano Barbacid and Stuart A. Aaronson at the NIH (12), and Robert Weinberg at MIT (13) identified Ras as the first human oncogene, and is now known to be mutated in at least 30% of all human cancers (14). Like all small GTPases, Ras sends signals when it is in the GTP-bound state. Importantly, many of the mutations that occur in Ras cause it to remain in the active, GTP-bound state, for an extended period of time resulting in an enhanced activation of its effectors. The importance of these mutations in promoting human cancer will be discussed in more detail below.

**Regulating the activities of small GTPases**

The activation of small GTPases is mediated by a group of proteins referred to as guanine nucleotide exchange factors (GEFs) (Figure 1.4). GEFs activate the small GTPases by facilitating the exchange of the bound GDP for GTP, and this occurs in response to signals such as those triggered by EGF. Specifically, the Ras protein is activated by a GEF called Son of Sevenless (SOS) (15,16), which is recruited to the EGF receptor when it is auto-phosphorylated on a specific tyrosine residue. The adaptor

protein Grb2 mediates the recruitment of SOS to the EGFR(17). The SH2 domain of Grb2 recognizes a specific phosphorylated tyrosine residue of Y-1068 on the EGFR (Tyr 1068) while the SH3 domains of Grb2 bind to SOS (18). Once recruited to the receptor, SOS is brought into close proximity to Ras which resides on the plasma membrane.

Although each GEF responds to specific signals that enable it to engage and activate its substrate GTPase, the basic mechanism underlying the ability of a GEF to stimulate GDP-GTP exchange is conserved. Specifically, GEFs induce at least two types of conformational changes in their target small GTPases that catalyze the dissociation of GDP and enable the loading of GTP. In the case of SOS, the first change induced in Ras causes the displacement of the switch 1 region, resulting in an 'opening-up' of the nucleotide-binding site. The second conformational change in Ras caused by SOS is a distortion of the switch 2 region that disrupts the essential interactions that occur between the phosphate group of the bound nucleotide and the coordinating $Mg^{2+}$, a key cofactor critical for high affinity binding of GDP. As a result, the affinity of the GTPase (in this case Ras) for the GDP is substantially weakened causing its dissociation from Ras. Because the concentration of GTP is 10-fold higher than GDP in cells, it is more likely to bind to the GTPase. The binding of GTP to Ras will then displace the GEF from the GTPase. The GTP-bound, or active form of the small GTPase is then able to carry-out specific functions in cells by engaging effectors.

As important as inducing the signaling capabilities of small GTPases at the right time and place, ensuring that they are only active for a finite time is also essential. However,

the small GTPases themselves have typically low intrinsic GTP hydrolytic activity, making it necessary for another protein to stimulate this reaction. This is achieved by a class of proteins called GTPase Activating Proteins or GAPs (Figure 1.4). Thus, GAPs play a critical role in the termination of signaling by the GTPases. GTP hydrolysis involves the nucleophilic attack of a water molecule on the γ-phosphate of GTP, requiring the proper orientation and polarization of the water molecule on the opposite site of the leaving group (i.e., GDP). GAP proteins function by stabilizing the transition state the GTP hydrolytic reaction. Typically, GAPs introduce a conserved arginine residue, known as 'the argnine finger', into the active site to stabilize the position of catalytic Gln (e.g., Q61 on Ras), and to neutralize the negative charges developing on the phosphates during the transition state for GTP hydrolysis.

The mechanism underlying how Ras, the first human oncogene identified, promotes cancer progression, underscores the importance of regulating the GTP binding/GTP hydrolysis cycle of small GTPases. Ras is frequently found to be mutated in human cancer. One of the most common spontaneously occurring mutations in Ras occurs at position 12 where a valine substitutes for a glycine residue. This mutation results in a Ras protein that has little GTP-hydrolytic activity (19,20). Thus, this mutant form of Ras is persistently active, resulting in the sustained activation of many mitogenic pathways, including the Ras-Raf-Mek-Erk pathway.

Cdc42 is a member of the Rho subfamily of the Ras superfamily of small GTPases (Figure 1.3). Like Ras, Cdc42 impacts mitogenic signaling, and its deregulation can also

lead to cellular transformation. Initially identified in a screen as an essential gene necessary for cell division in yeast, the Cerione laboratory cloned the human version of this protein in 1990 while searching for novel signaling partners of the EGF receptor (21). The role of Cdc42 in mammalian cell growth and cellular transformation was initially highlighted in a study that identified the product of the Dbl (for Diffuse B-cell lymphoma) as a GEF for Cdc42 (22). Dbl is the founding member of a family of oncogenic proteins which all function as GEFs for members of the Rho family of GTPases. Notably, these proteins commonly contain a tandem Dbl homology domain (DH domain) and Pleckstrin homology domain (PH domain), with the DH domain being responsible for the catalytic activity, and the PH domain mediating the appropriate cellular localization via its ability to interact with specific phospholipids (23). The fact that the ability of Dbl, when expressed in NIH3T3 fibroblasts, to induce cellular transformation was dependent on its GEF activity, suggested that Cdc42 may be involved in promoting malignant transformation.

Dominant-active mutant forms of Cdc42 have been discovered and examined for their ability to induce or promote transformation. The GTP hydrolysis-defective mutants, including the Cdc42 (G12V) or Cdc42 (Q61L) mutants, in certain cellular contexts, were capable of inducing transformation, but more often they were toxic to cells. Subsequently, a Cdc42 mutant (F28L) capable of spontaneously exchanging GDP for GTP while still being able to hydrolyze GTP (thus called a “fast-cycling” mutant) was generated and demonstrated to be strongly transforming when stably expressed in NIH3T3 fibroblasts (24). Thus, it seems that, similar to Ras, enhancing Cdc42’s signaling capabilities is

sufficient to drive transformation. However, distinct from Ras, Cdc42 needs to rapidly cycle between its GDP-bound and GTP-bound states in order to do so.

The mechanism underlying the ability of Cdc42 to induce cellular transformation also turns out to be different from how Ras drives transformation in that, unlike Ras, which transforms by activating its downstream effectors, Cdc42 prolongs the activation of the EGFR (25). Activated Cdc42 is able to form a complex with the E3-ubiquitin ligase Cbl, sequestering it from its substrate, the EGFR. This extends the half-life of the EGFR, sending mitogenic signals that drive cellular transformation. Consistent with this idea, inhibiting the EGFR using the EGFR kinase-inhibitor AG1478, is sufficient to block Cdc42-mediated cellular transformation, whereas cells transformed by oncogenic Ras are completely insensitive to this treatment.

As can be appreciated from the examples described above, the appropriate regulation of cell signaling events by the small GTPases has important consequences for normal cellular functions, and when de-regulated, in promoting disease states such as cancer. The Cerione group has long been interested in understanding how the activities of small GTPases are regulated. To this end, the laboratory discovered a protein called Cat as a potential mediator of mitogenic signaling. My thesis project is focused on understanding the role of Cat in cellular transformation. Cat is a GAP for the ADP-ribosylation factor (Arf) family of small GTPases, and a scaffold protein that can interact with a variety of signaling molecules. I will first provide some background information on ArfGTPases

and ArfGAPs, and then I will focus on the known cellular functions of Cat in a later section.

**Arf GTPases**

Arf, originally identified as a cofactor required for the cholera toxin-catalyzed ADP-ribosylation of the α subunit of the heterotrimeric G protein Gs, is a member of a Ras superfamily that has been primarily implicated in membrane trafficking and regulating organelle structure (Figure 1.3). In mammals, the Arf family is subdivided into three classes based on their primary amino acid sequence similarities; Arf1, Arf2, and Arf3 comprise Class I, Arf4 and Arf5 comprise Class II, and Arf6 is the sole member of Class III (26,27). At least one member in each of these classes is conserved in all metazoans, whereas yeast lack only the Class II members of the Arf GTPases. Like other small GTPases, Arf GTPases bind to guanine nucleotides, and their activity is regulated by the GTP-binding / GTP-hydrolysis cycle. The binding of GTP by Arfs and its subsequent hydrolysis are regulated by a family of GEFs and GAPs. The GEFs commonly contain Sec7 domains, which correspond functionally to the DH domain found in the Dbl family of Rho GEFs. The Sec7 domain stimulates GDP release from the Arfs, allowing GTP to then bind to the protein. Arf GAPs catalyze the hydrolysis of GTP to GDP in Arf GTPases, which is critical because Arf GTPases exhibit no detectable intrinsic GTP hydrolysis activity (28). Arf GAPs have a conserved zinc-finger catalytic domain that contains the conserved arginine finger that is also found in Rho GAPs. Like other Ras superfamily members, Arf GTPases are post-translationally modified with lipids to help them bind to specific membrane compartments. However, Arf GTPases are uniquely

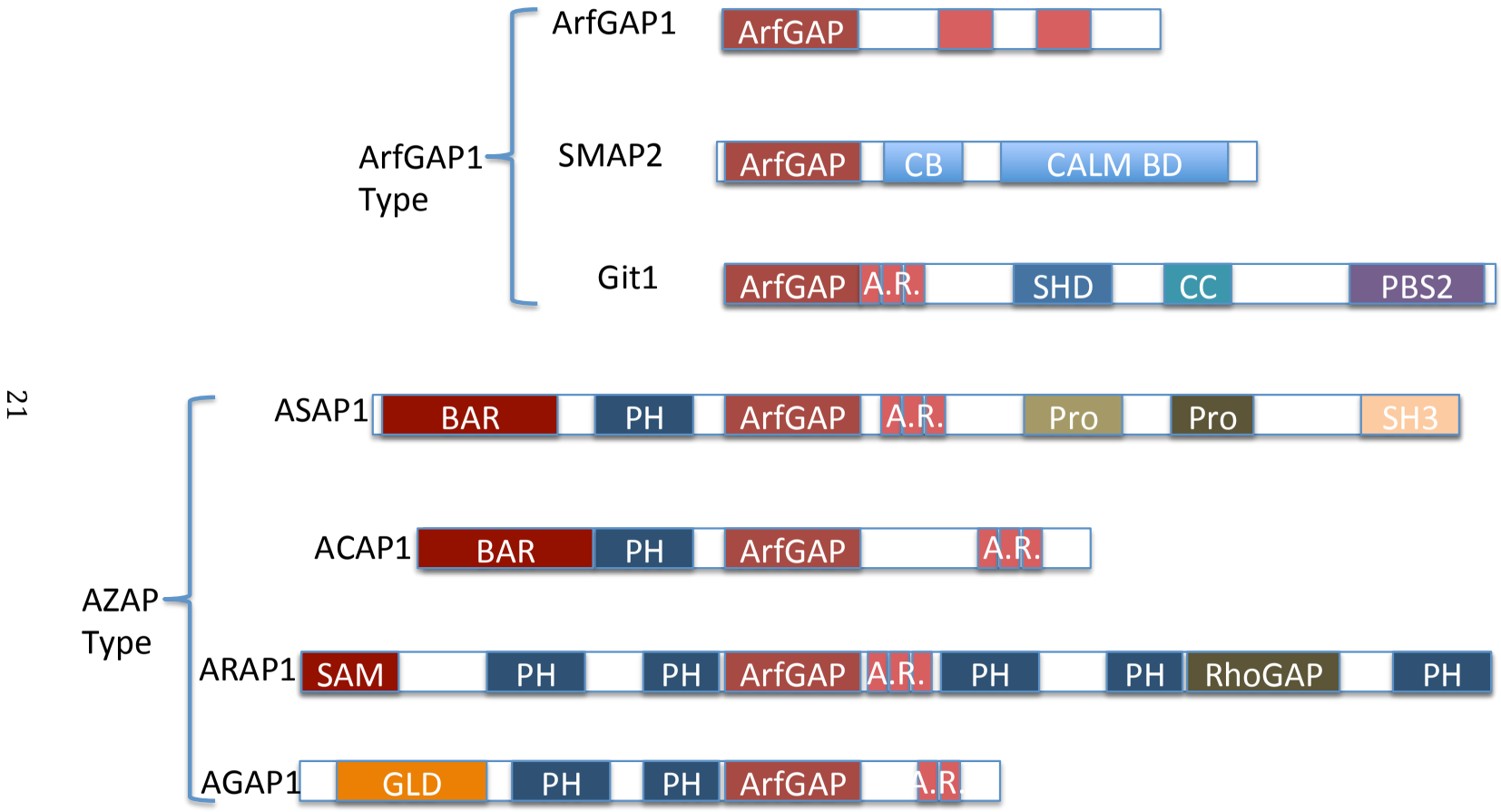
myristolated at their N-terminus as opposed to being geranylgeranylated or farnesylated at the C-terminus like most other small GTPases (29).

**Arf GAPs**

Arf GAPs are a family of proteins that are ubiquitously expressed in eukaryotes and regulate the activation of Arf GTPases. Members of the family share a conserved functional domain, which includes a signature zinc-binding motif (CX2CX16CX2CX4R) that has a structural, rather than a catalytic role (30). There are at least 32 genes in humans that encode proteins with Arf GAP domains and they can be categorized into two types according to their protein structure (Figure 1.5); Arf GAP1 type and AZAP type. The major distinguishing feature of these two groups has to do with the location of the Arf GAP domain. Members of the Arf GAP1 group have their Arf GAP domain located at the N-terminus of the protein, while the AZAP type of GAPs has the Arf GAP domain between the PH and ankyrin repeat domains (31). Arf GAP1-type proteins include ArfGAP1, SMAP, and Cat. The AZAP-type group includes ASAP, ARAP, ACAP and AGAP. A majority of the Arf GAPs are multidomain-containing proteins with molecular weights that range from 80 kDa to 200 kDa.

Because Arf GTPases have undetectable intrinsic rates of intrinsic GTP hydrolysis, the manifestation of this activity relies solely on the binding of an ArfGAP. However, as the complex domain structure of the Arf GAP proteins might suggest, their cellular roles often involve additional functions through their ability to bind to several other signaling

**Figure 1.5 Domain structures of the ArfGAP family.** Members of the ArfGAP family catalyze GTP-hydrolysis by Arf GTPases. The ArfGAP domain is the catalytic domain which contains the signature zinc-binding motif and the catalytic 'arginine finger'. ArfGAPs can be categorized according to their domain composition. They are subdivided depending on whether the Arf GAP domain is located at the N-terminus, or is situated between the Pleckstrin Homology domain and the Ankyrin Repeats (Adapted from (31)).


ArfGAP1
Type
ArfGAP1
ArfGAP
SMAP2
ArfGAP
CB
CALM BD
Git1
ArfGAP
A.R.
SHD
CC
PBS2
AZAP
Type
ASAP1
BAR
PH
ArfGAP
A.R.
Pro
Pro
SH3
ACAP1
BAR
PH
ArfGAP
A.R.
ARAP1
SAM
PH
PH
ArfGAP
A.R.
PH
PH
RhoGAP
PH
AGAP1
GLD
PH
PH
ArfGAP
A.R.

proteins. In support of this idea, there are many instances where the abilities of the Arf GAPs to associate with their binding partners are unrelated to their GAP function (31)

In fact, Arf GAPs are able to interact with numerous proteins that include vesicle cargo/coat/adaptor proteins, lipid-modifying enzymes, and protein kinases. Evidence is accumulating that Arf GAPs function as more than just terminators of Arf GTPase activity but also as signaling effectors where they work as scaffolds, coordinating the interactions between multiple proteins to efficiently carry out the functions of the activated Arf GTPases (32). One of the best examples of this came from a study performed in yeast where Arf GAPs were identified in a screen for suppressors of loss of Arf GTPase function (33). If Arf GAPs were solely acting as terminators of Arf GTPase-signaling, then over-expressing Arf GAPs in yeast would not have resulted in the rescue of the loss of Arf GTPase phenotype seen in this study. Thus, it is becoming clear that Arf GAPs can influence the functions of Arf GTPases on a number of levels. Indeed, the Arf GAP, Glo3, has been shown to facilitate the formation of an Arf1-mediated priming complex that is composed of an activated Arf GTPase, coatamer, and cargo molecules (34). Also in humans, two GAPs for Arf1, namely ArfGAP2 and ArfGAP3, interact with the COP I and SNARE proteins and participate as essential factors for vesicle formation, working as downstream effectors for Arf1 (35). In fact, a similar mode of regulation where GAPs act to both potentiate and terminate GTPase signaling, has been suggested for GPCR signaling where RGS proteins act as both signal amplifiers and signal terminators (36,37).

**Identification of Cat**

Cat, also referred to as Git (for G-protein coupled receptor kinase interactor) or PKL (for paxillin kinase linker), is a multifunctional protein that is known to regulate the activity of certain members of the Arf family of small GTPases, as well as function as a scaffold that brings various proteins together via its numerous protein-binding domains (38). Cat is conserved from *C.elegans* to humans (39). All mammals studied thus far express two Cat homolog proteins; Cat-1 and Cat-2. The amino acid sequences of these homologs are 65% identical and 85% similar. The expression profiles of these proteins have been studied in mice, using a 'gene-trap' approach (40), where a reporter gene (i.e., beta-galactosidase) was inserted into an exon of an endogenous gene of interest, and when expressed results in the expression of a fusion protein consisting of parts of the endogenous protein and a reporter protein that can be readily detected. The expression of such a fusion protein, in turn, is under the regulation of the endogenous promoter for the gene of interest, thus allowing for a means to read out where a gene of interest is expressed in a model system (i.e., in a developing mouse embryo). In the Cat-gene-trap experiments carried-out in mice, Cat-1 was shown to be highly expressed in the brain, blood vessels, and mature spermatids, whereas Cat-2 was expressed more ubiquitously throughout the entire organism (40). Another distinction between Cat-1 and Cat-2 has to do with their alternative splicing. Unlike Cat-1, Cat-2 is alternatively spliced extensively resulting in more than 10 splice variants and 32 different transcripts (41). Except for 'Cat-2 short', a splice variant that was identified in fibroblasts as a protein that co-precipitated with paxillin, very little is known regarding the functions of these Cat-2 isoforms (42).

Interestingly, the Cat-1 and Cat-2 proteins, that we often collectively refer to as Cat, were identified more than a decade ago through three independent lines of investigation as binding partners for three distinct proteins. In one such study performed by our laboratory, the Cat proteins were identified in pull-down assays as phosphorylated proteins that bound to Cool-1, a GEF for Cdc42 (38), whereas in the Lefkowitz laboratory these same proteins were named Gits (for G protein-coupled receptor kinase-interacting protein), because they were discovered through their ability to bind to G-protein coupled receptor kinase 2 (GRK2) (43). The Turner group then identified Cat-2 (which they called PKL for Paxillin-Kinase-Linker) as a binding partner for paxillin, an important signaling molecule involved in cell migration (44). Protein sequencing performed on Cat-1 and Cat-2 showed that they contained an Arf GAP domain, and the Lefkowitz laboratory demonstrated that they could function as GAPs for the Arf GTPases (45). However, it is also worth emphasizing that the ability of the Cat proteins to interact with a variety of other proteins, (i.e., Cool, GRK, and paxillin) also highlights that they are multi-functional and participate in a number of cellular processes. For example, Cat-Cool interactions possibly point to an interesting connection between a role for Cat in coordinating Arf GTPase activity with Cdc42 signaling, whereas the interactions between Cat and Grk, discovered in the Lefkowitz laboratory, suggest a role for the Cat proteins in receptor internalization. Moreover, the identification of Cat as a binding partner for paxillin was the first clue that Cat may also have a role in focal complex regulation.

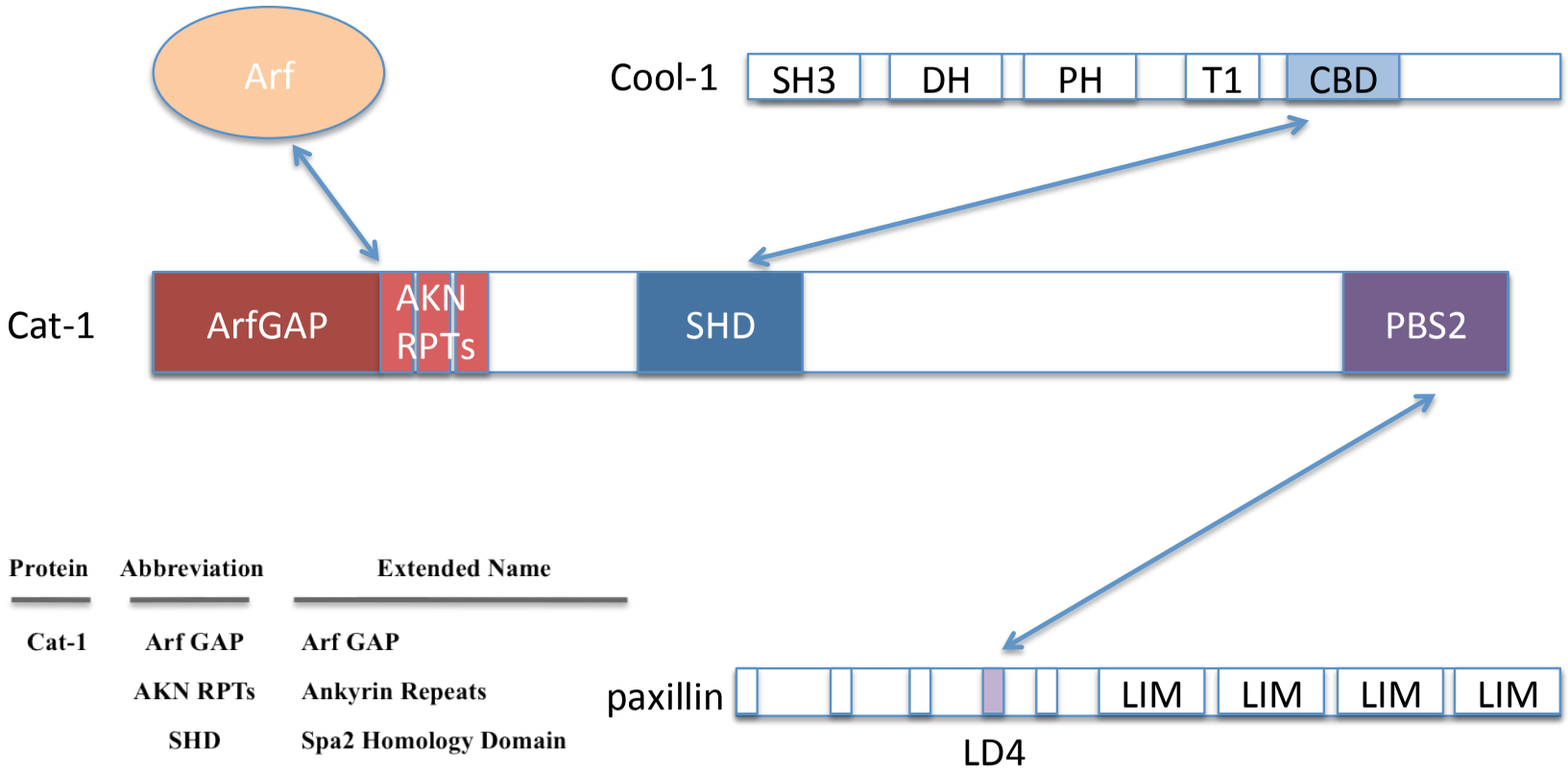
Indeed, since their discovery 10 years ago, the Cat proteins have been implicated in the regulation of various cellular processes such as neuronal dendritic spine formation

(46), neuronal synaptic activity (47), and T cell activation (48). However, the molecular mechanisms that have been proposed to explain their roles in mediating these diverse cellular functions have mostly centered around how Cat functions as a scaffold and affects cell morphology. For example, the localization of the Cool-Pak complex to focal adhesions through Cat-paxillin interactions was shown to be important for focal complex regulation and thus, cell morphology (49-51). However, there are reasons to suspect that Cat may have additional roles in cells. For instance, Cool-1, a major binding partner for Cat, has been shown to have a central role in Cdc42-mediated as well as Src-dependent cellular transformation (52,53). Moreover, Cat was shown to regulate the internalization of G-protein coupled receptors, as well as the internalization of EGFRs (54), raising the possibility that Cat may also impact mitogenic signaling.

**Domains and interacting partners of Cat**

Since its identification, there have been significant advances in our understanding of the structural domains in Cat that mediate its cellular functions. As depicted in Figure 1.6, Cat is a 95 kDa protein that contains multiple protein-interacting domains including an Arf GAP domain at the N-terminus, followed by a Spa2 Homology domain, a Coiled-coil domain, and a Paxillin binding sequence/Focal Adhesion Targeting homology at the C-terminus. Each of the domains and the roles they play in Cat-mediated signaling events will now be covered below.

**Figure 1.6 Cat is an Arf GAP protein and a scaffold that interacts with Cool, a GEF for Cdc42/Rac, and paxillin, a focal complex protein that is involved in integrin-signaling.** Cat is an Arf GAP with its Arf GAP domain located at the N-terminus. It is able to bind to Cool, a GEF for Cdc42 and Rac, through its Spa2 Homology Domain (SHD). Cat also binds to paxillin, an important focal complex protein that mediates integrin-dependent signaling and is also involved in cell migration, through its Paxillin Binding Sequence 2 (PBS2) domain.




| Protein | Abbreviation | Extended Name |
|---|---|---|
| Cat-1 | Arf GAP | Arf GAP |
| | AKN RPTs | Ankyrin Repeats |
| | SHD | Spa2 Homology Domain |
| | PBS2 | Paxillin Binding Sequence 2 |
| Cool-1 | DH | Dbl Homology |
| | PH | Plekstrin Homology |
| | T1 | T1 |
| | CBD | Cat Binding Domain |
| Paxillin | LD4 | LD motif 4 |
| | LIM | Lin11, Isl-1, Mec-3 |

***Arf GAP domain***

The Arf GAP domain lies within the N terminus of Cat. Arf GAP domains, in general, are unique compared to Rho GAPs in that they have a cysteine-rich, zinc-binding region with the binding of zinc having a role in promoting protein-folding rather than a direct catalytic function (30,31). Also, the molecular mechanism underlying Arf GAP-catalyzed GTP-hydrolysis was initially thought to be different from that used by Rho GAPs. This was based on the crystal structure of the complex between Arf1 and ArfGAP1 (55), which showed that the catalytic arginine was remote from the active center of Arf1 prompting the authors to propose a mechanism where coatamers provide the critical arginine and cooperate with ArfGAP1 to mediate catalysis. However, biochemical studies have consistently reported that the catalytic arginine of Arf GAPs is important for catalysis, and recent structural data of a complex between ASAP3, an Arf1 GAP, and its substrate Arf1 by Wittinghofer and colleagues confirmed that the invariable 'arginine finger' is indeed located at the active site (56). Currently, the general consensus in the field is that the arginine finger in an Arf GAP functions in a similar manner as in other Rho or Ras GAPs.

The molecular mechanisms that regulate Cat's Arf GAP activity are not well understood. In fact very little is known about the regulation of Arf GAP activities in general. There are a few proposed mechanisms for the regulation of Arf GAP1, the most well characterized of the Arf GAPs, but a consensus has proven to be difficult to be reached (57) . GTPase activation by Arf GAP1, for example, was proposed to be dependent on coatamers and inhibited by cargo. However, it has also been suggested that

ArfGAP1 senses the curvature of a vesicle, and GTPase activation by the GAP occurs only when the curvature exceeds a certain level. To date, there has been only one study, carried out by the Premont laboratory, that has investigated the regulation of Cat's GAP activity (45). It focused on the relationship between the phosphorylation states of phosphatidylinositols (PIPs) and Cat's GAP activity. This was because, at the time this study was performed, PIPs had been shown to regulate ArfGAP1 activity (58). The report shows that Cat's GAP activity is stimulated by phosphatidylinositol 3,4, 5-trisphosphate (PIP3), but not by PIP2 or PIP, and was unique in that, unlike ArfGAP1 which works on Arf1 Arf2, Arf3 (class I Arfs), and Arf5 (a class II Arf), Cat was shown to work on all three classes of Arf GTPases including Arf6 (a class III Arf) *in vitro*. These findings, and the fact that Cat and Arf6 co-localize in cells, are the main reasons for the suggestions that Cats acts as a GAP for Arf6. An interesting idea that stems from the study showing that the GAP activity of Cat is stimulated by PIP3, is that activated Arf6 is known to activate PIP5K to produce PIP2 (59), which, in turn, is the starting material to generate PIP3. Thus, a potential negative feedback loop may exist between activated Arf6 and the GTP hydrolysis activity of Cat.

***Ankyrin-Repeats domain***

Next to the GAP domain in Cat are the ankyrin-repeats. These are common motifs found in proteins that mediate numerous protein-protein interactions. Judging from the extensive interface that is formed by the Arf GAP domain and the ankyrin repeat domain in the crystal structure of PAPbeta2, another Arf1 GAP, the ankyrin repeat is thought to add structural integrity to the GAP domain (30). It should also be noted that the ankyrin

repeat of Cat was able to make an intramolecular interaction with the SHD domain within Cat, and disabling this intramolecular interaction using a mutant form of Cat resulted in enhanced intermolecular interactions with paxillin, as well as interactions mediated by the C-terminal PBS domain of Cat (60). Thus, the interactions between the ankyrin-repeats and the SHD domain were auto-inhibiting the interactions between Cat and paxillin. Interestingly, auto-inhibitory interactions within ASAP1 also regulated its binding to Arf GTPases and its Arf GAP activity (61). It will be interesting to see whether a similar mechanism exists for Cat where its GAP activity is regulated by the same intramolecular interaction that regulated its interaction with paxillin.

***SHD domain***

C-terminal to the ankyrin-repeats in Cat is the Spa homology domain (SHD). Cat is the only mammalian protein known to contain this domain. In fact, the only other proteins identified that have SHD domains are the members of the Spa2 family of proteins in yeast (51). Interestingly, Spa2 proteins function in yeast by interacting with cell polarity proteins, and are essential for the polarized growth of yeast (62). The SHD domain in Cat is responsible for its ability to form a tight binding interaction with Cool-1, which is a GEF for the small GTPase Cdc42 (51). Thus, the Cat-Cool complex in cells brings together an activator of Cdc42 and a negative regulator of Arf6, providing a signaling node that coordinates the activities of the two small GTPases, Cdc42 and Arf6 (61).

It has been recently suggested that the interaction between Cool and Cat is regulated by the phosphorylation – de-phosphorylation cycle of Cool (63). As will be discussed later, it was proposed that the phosphorylation of Cool by tyrosine kinases such as Src promote the dissociation of Cool and Cat. This model was used to explain why Src-promoted migration was dependent on Cool.

The SHD domain also mediates the interaction of Cat with Phospholipase Cγ (64). PLCγ plays an important role in inositol signaling, as it catalyzes the production of Di-Acyl Glycerol (DAG) and IP3 from phospholipids. IP3 then diffuses throughout the cytosol and binds to IP3 receptors expressed on the surfaces of the ER. Activation of the IP3 receptors, which function as $Ca^{2+}$ channels, mediate the release of $Ca^{2+}$ stores from the ER into the cytosol, which, in turn, initiates a myriad of signaling cascades that regulate many cellular activities including exocytosis and apoptosis. Cat's ability to bind PLCγ was reported to be important for promoting PLCγ's hydrolytic activity. Moreover, the phosphorylation state of PLCγ is also known to activate its catalytic activity. Interestingly, one study showed that ectopically expressing a mutant form of Cat that was lacking its SHD domain (and therefore could not bind to PLC γ) in cells, reduced the level of phosphorylated PLCγ compared to WT Cat (64). This suggests that the binding of Cat to PLCγ may help maintain it in a phosphorylated or active state.

***PBS2 domain***

Downstream of the SHD domain in the primary sequence of Cat-1, near its C-terminus, is a paxillin-binding sequence 2 (PBS2) domain. Actually there are two

identified paxillin-binding sequences in Cat, and they are homologous to the paxillin-binding regions found in other proteins such as the Focal Adhesion Kinase (Fak) (44). The first sequence, PBS1, is located immediately C-terminal to the ankyrin-repeats. PBS1 has been reported to mediate paxillin interactions with Cat-2-short, an alternative splice variant of Cat-2 that is truncated at its C-terminal end. PBS2 of both Cat-1 and Cat-2 has been shown to bind to paxillin, and Small Angle X-ray Scattering (SAXS) experiments performed on the PBS2 domain of Cat suggest that it assumes a similar fold as the homologous sequence in Fak (65). Using this similarity, the Premont laboratory has been able to identify point mutations in this domain that specifically block the ability of Cat to interact with paxillin. Interestingly, the C-terminal end of Cat, where the PBS2 domain is located, has also been shown to be important for Cat to interact with many additional proteins including, Hic-5, huntingtin, liprin-alpha and eNOS (66),(67),(68).

***ArfGAP domain-interacting proteins : Arf1 and Arf6***

The Arf GAP domain of Cat has been shown to stimulate GTP hydrolysis in all three classes of Arf GTPases *in vitro* (45). However, cellular studies where Cat's GAP activity was functionally disabled through mutation suggested Arf1 and Arf6 to be physiological substrates of Cat (42,69).

Arf1 is best known for its role in regulating secretory membrane transport at the Golgi, where it mediates the trafficking between the Golgi and ER, as well as trafficking between Golgi cisternae. GTP-bound Arfs are bound to Golgi membranes and recruit coat protein complex I (COP I), clathrin AP (adapter proteins) coats, and GGAs (Golgi-

associated γ-adaptin ear homology domain Arf-interacting proteins) to budding transport vesicles (27). These coat proteins help sort cargo that will be carried into vesicles and trafficked between the ER and Golgi. While the role of Arf1 as being a critical mediator in recruiting coatamers to trafficking vesicles was initially identified through *in-vitro* biochemical studies, the *in-vivo* functions of Arf1 in vesicle-trafficking have been confirmed using the dominant-negative GTP binding-defective form of Arf1 (T27N) as well as the dominant-active, GTP-hydrolysis-defective mutant (Q71L). In particular, over-expressing Arf1 (T27N) resulted in the inhibition of vesicular export from the ER to Golgi, and the release of β-COP to the cytosol, whereas the expression of Arf1 (Q71L) resulted in the accumulation of vesicles in the Golgi, with β-COP remaining attached to Golgi membranes (70). Despite these extensive studies on the Arf1 GTPase and its role in Golgi trafficking, there are still outstanding questions that need to be answered regarding the exact mechanism of recruitment of Arf1 to the Golgi membrane, and the role of Arf1 GTP-hydrolysis in vesicle formation and fission (57). Recently, it was shown that, depending on the cellular context, Arf1 is also found at the plasma membrane where it may have a role in clathrin and dynamin-independent, Cdc42-mediated pinocytosis. However, much additional work is needed to fully understand the role of Arf1 at the plasma membrane (71).

Arf6 is localized at the plasma membrane where it has been reported to regulate membrane transport (72,73),(74). Arf6 has been shown to be involved in a subset of both clathrin-dependent and clathrin-independent endocytosis. Unlike Arf1, Arf6 has not been shown to directly recruit coat proteins. However, Arf6 can activate PIP5K as well as

Phospholipase D (PLD) (59),(75), and its role in endocytosis has been explained through its ability to affect lipid metabolism. For example, PIP2, which is a product of PIP5K activation, can recruit clathrin coats to the plasma membrane. Interestingly, PIP5K is not only a downstream effector of Arf6, but also uses phosphatidic acid (PA) as a cofactor, which is, in turn, a product of PLD, another effector of Arf6 (26). So far, Arf6 has been shown to affect GPCR endocytosis through clathrin-dependent endocytosis, and also MHC class I proteins, M2-muscaranic acetylcholine receptors, and beta1-integrins through clathrin-independent, caveolae-independent endocytosis (26,76).

Arf6 has also been suggested to participate in the recycling of endosomes (77). This was first shown in CHO cells where over-expressing either wild-type Arf6, or Arf6 mutants defective in their ability to hydrolyze GTP (Q71L) or bind GTP (T27N), interfered with the recycling of transferrin receptors. Whereas, over-expressing wild-type or constitutively-active Arf6 slowed the uptake of the transferrin receptors from the cell surface, resulting in their accumulation at the plasma membrane, the over-expression of dominant-negative Arf6 resulted in the intracellular distribution of the receptors due to the interference with their recycling. While transferrin receptors traffic through a clathrin-dependent mechanism, Arf6 was later shown to also affect the recycling of proteins that lacked the classical clathrin-dependent sorting signals, such as, MHC class I proteins and GPI-anchored proteins (78). This role in recycling by Arf6 has also been shown to be dependent on its effects on PLD activity, as Arf6 mutants that are unable to stimulate PLD blocked recycling and resulted in accumulation of tubular endosomes (79).

Arf6 is also known for its ability to affect actin structures at the cell periphery manifested as membrane ruffles, and cell spreading. One of the pathways that has been suggested to be responsible for Arf6-mediated membrane remodeling involves the Arf6-mediated activation of Rac1. As will be discussed below, the Arf6 GEF, ARNO, mediated the link between Arf6 activation and Rac activation, by recruiting DOCK180, a Rac GEF to the plasma membrane leading to lamellipodia formation (80). However, this is not the only pathway that links Arf6 to Rac activation. NM23-H1, a nucleoside diphosphate kinase (NDK) that phosphorylates GDP, was found to be an Arf6 effector that sequesters Tiam1, a Rac-specific GEF, away from its substrate Rac (81). Thus, activated Arf6 led to a decrease in the level of activated Rac in polarized epithelial MDCK monolayers. This pathway, where activated Arf6 leads to the de-activation of Rac, was suggested to mediate Arf6-dependent disassembly of adherens junctions between cells, where actin filaments built up by activated Rac promote the stability of adherens junction structures.

In this context, it is worth noting that Arf6 was shown to localize at invadopodia, an actin-membrane structure that is thought to be utilized by cancer cells in invasion and metastasis (82),(83). Moreover, Arf6 activity was crucial in mediating matrigel-invasion by MDA-MD-231 breast cancer cells. Amongst many potential GEFs for Arf6, GEP100 specifically mediated this function of Arf6, which, in turn, bound to phosphorylated EGFRs directly (84).

***Cool***

Cool (for Cloned-out-of-library) is a member of Dbl family of GEFs, activating Cdc42 and Rac1 (85). It was co-discovered by the Cerione laboratory (86) and by Manser, Lim and colleagues (and named Pix for Pak-interacting exchange factor) (87) as a p21-activated kinase (Pak)-binding partner from a yeast two-hybrid screen. Cool's ability to regulate Pak activity is dependent on the presence of a short domain called T1, located C-terminal to the tandem Dbl Homology domain-Pleckstrin Homology, a signature domain for Dbl family of GEFs, and upstream of the Cat-binding domain (CBD) located at the C-terminus. The presence of the T1 domain negatively regulates Pak activity, by inhibitng the GEF activity of the Cool proteins. This auto-inhibitory effect can be reversed by the phosphorylation of p85 Cool-1 (Cool-1 from now on) at tyrosine 442 located immediately downstream to the T1 domain (53). When Cool-1 is phosphorylated at Tyr442, its GEF activity is activated.

The phosphorylation of Cool-1 influences another function, namely its ability to act as an 'effector' for Cdc42. Thus, phosphorylated Cool-1 binds with high affinity to the activated, GTP-bound form of Cdc42. Thus Cool-1 not only functions as an upstream regulator of its substrate, but also act as a downstream effector (52,53).

Cool has been implicated in many cellular activities, and our laboratory has shown that it is a critical mediator of Cdc42-promoted cellular transformation (52). The Cdc42 (F28L) mutant is capable of the spontaneous exchange of GDP for GTP, while maintaining its GTP hydrolytic activity. Its expression in NIH3T3 cells induced cellular

transformation (24). The mechanism behind such transformation involves Cool-1. The activated, GTP-bound Cdc42(F28L) mutant is able to bind to Cool-1 which, in turn, associates with c-Cbl, an E3 ubiquitin ligase that negatively regulates EGF Receptor (EGFR)-signaling by initiating its degradation. The Cdc42-Cool-1-Cbl complex sequesters Cbl away from the EGFR, prolonging its signaling lifetime.

***paxillin***

Paxillin is a scaffold protein that is involved in integrin-mediated signaling. It contains multiple protein interacting domains; specifically 5 LD domains in the N-terminal half of the protein, and 4 LIM domains in the C-terminal half (Figure 1.6). These domains also contain a number of potential tyrosine and serine/threonine phosphorylation sites, and they appear to mediate complex, protein-protein interactions that coordinate many different signaling events, making paxillin a central node in integrin-mediated signaling. As such, paxillin has been shown to affect cell motility, cell survival, invasion, and cell growth (88).

The LIM domains in paxillin are responsible for its localization to focal adhesions (FAs) (88). Several of the phosphorylations of serine and threonine residues within these domains facilitate the localization (89). PTP-PEST, a phosphatase that has been shown to play an important role as a tumor suppressor in triple-negative breast cancers by keeping multiple proto-oncogenic tyrosine kinases in check (90) as well as participating in cell migration, is recruited to paxillin via an interaction with the LIM domains and functions

to de-phosphorylate Tyr118, which, in turn, has been shown to be important in focal complex regulation and cell survival (91),(92).

Interestingly, on top of being a localization motif for focal adhesions in paxillin, the LIM domains of the paxillin-related homolog Hic-5 or Death-Associated Lim-only Protein (DALP), have a role in apoptosis (93). Hic-5 is a close family member of paxillin, as is DALP although it only contains the 4 LIM domains. When C2C12, a mouse myoblast cell line, was selected to stably express either Hic-5 or DALP and then induced to undergo differentiation, these cells underwent rapid apoptosis. The specific study that elucidated the role of DALP and Hic-5 as apoptotic factors initially suggested a mechanism for how these proteins can contribute to programmed cell death in differentiation. It also provided hints as to how these LIM domain-containing proteins that are homologous to Hic-5 and paxillin can negatively impact cell growth (94,95).

The LD domains of paxillin, especially the LD4 domain, mediate the ability of paxillin to interact with Cat1 (44). This interaction localizes the Cat-Cool-Pak complex to paxillin, which in turn, affects cell spreading. Such localization of Cat to paxillin would coordinate the localized activation of Rac, and the resulting morphological changes that this GTPase triggers (49). The LD2 and LD4 domains have also been shown to interact with FAK which regulates ERK activity and survival (88).

Tyrosine phosphorylation of paxillin has been shown to have roles in mediating various interactions. Tyr31 and Tyr118, which lie within the N-terminus where all four

LD domains are located, are the main phosphorylation targets of the Fak/Src tyrosine kinases. These phosphorylation sites have been shown to interact with either the CrkII-Dock180-ELMO complex which can promote the activation of Rac and Arf6 (96,97), or with p120RasGAP which leads to activation of p190RhoGAP and the de-activation of Rho activity (98). Both of these interactions have been implicated in regulating Rho GTPase-mediated focal adhesion maturation and protrusion. These tyrosine residues have also been shown to be important in apoptosis although the mechanism is unclear.

**Known cellular functions of Cat**

Cat has been shown to exert diverse cellular functions, mainly through its ability to interact with several different proteins.

***Cat in focal complex regulation and migration in fibroblasts***

The first indication that Cat can promote cell migration came from an observation that over-expressing Cat in NIH3T3 cells or COS-7 cells enhanced the migration rates of those cells as read-out by time-lapse microscopy or in Boyden chamber assays (51). It was also found that co-expressing Cat and Cool together in cells led to the disassembly of focal complexes, and this was accompanied by increased interactions between Cat and paxillin. Thus, the interactions between Cat and paxillin were suggested to cause focal complex disassembly.

Our laboratory has also recently implicated Cat in the Src-stimulated migration of fibroblasts (63). We have shown that the tyrosine phosphorylation/de-phosphorylation

cycle of the Cdc42 GEF, Cool-1, regulates the interaction between Cat and paxillin which, in turn, drives focal complex assembly and dis-assembly in Src-transformed NIH3T3 fibroblasts. More specifically, the tyrosine phosphorylation of Cool-1 by Src was suggested to weaken the Cool-Cat interaction, while at the same time, enhance the Cat-paxillin interaction. The enhanced Cat-paxillin interaction was proposed to promote a more dynamic focal complex assembly and disassembly cycle, stimulating the migration and invasive activity exhibited by these cells.

There have also been reports that describe a role for Cat in cell spreading and, in particular, in the formation of lamellipodia. Two mechanisms have been proposed to account for this function of Cat. One mechanism has the Pak kinase, which binds to Cat, acting as an upstream activator of Rac (99). Thus, when Cat binds to paxillin, Cat is localized to the focal complexes and the Rac-mediated protrusion becomes limited. This model is supported by the observation in cells where the over-expression of a paxillin mutant that lacks the LD4 domain, which mediates the Cat-paxillin interaction, led to Rac activation and membrane protrusion (49).

Another mechanism explains the effects of Cat on cell spreading through Cat's Arf-GAP activity. That is, the GAP activity of Cat catalyzes GTP hydrolysis by Arf6, which, in turn, influences lipid trafficking (100). In this study, it was observed that a Cat mutant that lacked the Arf-GAP domain promoted spreading, and this effect was inhibited by a dominant-negative form of Arf6, and also by dominant-negative Rac. Dock180, a GEF for Rac and a known effector for Arf6, has been suggested to explain the molecular link

between Arf6 and Rac (80). That is, activated Arf6 can localize and directly turn-on the GEF activity of Dock180 toward Rac. In fact, the Ginsberg laboratory found that an integrin-paxillin complex inhibited stable lamellipodia formation by blocking Rac activation and this was dependent on the recruitment of Cat to paxillin (50).

***Cat's role in influencing neuronal cell morphology and in neuronal synaptic activity***

Similar to studies performed in fibroblasts that showed that Cat can impact cell shape and migration, the Horwitz laboratory found that Cat-1 can also influence neuronal cell morphology (46). In doing so, they identified the Synapse Localization Domain on Cat, which lies between the SHD and PBS domains, and it seems to be responsible for localizing Cat to synapses in hippocampal neurons. Neurons that over-express SLD disrupted the ability of the endogenously expressed Cat to be localized to the synapses, resulting in defective spine morphology and dendritic synapse formation in neuronal cells. Interestingly, these defects were rescued when an activated form of Rac was ectopically expressed in the neurons, suggesting again that Cat can influence cell morphology through a Rac-dependent pathway.

Other laboratories have shown that it was the human Scribble (hScrib) protein that is responsible for targeting Cat to synapses through its interaction with Cool (101). Scribble is a membrane-associated scaffold that was first identified as a tumor suppressor in *Drosophila* (102). It has since been shown to be required for maintaining cell polarity in various cell types. Scribble is localized to the plasma membranes because its Leucine Rich Repeats (LRR) motif interacts with plasma membranes and its PDZ domain can

interact with its binding partners, such as Cool (101). Once at the point of synaptogenesis, $Ca^{2+}$ can activate Cool through a Calmodulin-dependent kinase pathway. Kinases involved in Calmodulin-dependent kinase pathways, namely CaMK and CaMKK, were shown to phosphorylate Cool-1 at Ser516 site in a $Ca^{2+}$ dependent manner in hippocampal neurons, which in turn led to the activation of Cool's GEF activity. Activating Cool led to the activation of Pak which was necessary for synaptogenesis (47).

Attention deficit hyperactivity disorder (ADHD) has been attributed to defects in Cat-1 function (103). Through genomic studies, ADHD-associated Single Nucleotide Polymorphism (SNP)s were found in the Cat-1 gene in humans. When Cat-1 knock-out mice were generated, they displayed behaviors characteristic of ADHD. The Cat-$1^{-/-}$ mice showed much higher locomotive activity in open-field tests. In fact, treating these Cat-$1^{-/-}$ mice with amphetamine and methylphenidate, psychostimulants commonly used to treat ADHD, reversed the hyperactivity in these mice. Cat was shown to be necessary for inhibitory transmission at presynapses in hippocampal neruons. The molecular mechanism behind the $Cat^{-/-}$-mediated manifestation of hyperactivity was attributed to reduced expression levels of Cool-1/Cool-2 in the $Cat^{-/-}$ neurons which resulted in less Pak3 activity. However, the exact mechanism behind how the loss of Pak3 activity specifically affected inhibitory transmission in the presynapse is not yet understood.

Separately, Cat-1 was shown to bind Huntingtin(htt), a pathogenic protein for Huntington's Disease (HD) and this interaction was correlated with the pathogenicity of the disease (67). The C-terminus of Cat-1 was found to interact with htt in a yeast two-

hybrid screen. When the C-terminal Cat-1 fragment was co-expressed with an N-terminal htt fragment containing 68 glutamine residues (HD169Q68) in HEK293 cells, htt aggregates were formed to a 3-fold greater extent in the same time period as the amount of aggregates formed in HD169Q68-only expressing cells. Accordingly, knocking-out endogenous Cat-1 by siRNA dramatically slowed the formation of htt-aggregates. In fact, the C-terminus of Cat-1 and the N-terminus of htt interacted in cells, and high amounts of the C-terminal, truncated Cat-1 were found in the brains of patients suffering from Huntington's disease.

***Cat in polarity***

One common system that is often used to study cell polarization is the wound-healing assay. When a scratch, or 'wound', is introduced onto a monolayer of cells grown in culture with a sharp, narrow-ended object like a pipette tip, the cells that are lining up against the wound undergo a drastic, but polarized, change in morphology and coordinately start migrating towards the freshly made gap. Cdc42 has been implicated in establishing such polarity within migrating cells. The Hall laboratory suggested that this function of Cdc42 is achieved through a two-fold mechanism (104). The first is the re-orientation of the Golgi and centrosome towards the leading edge of the cells (105), and the second is the polarized protrusion of the leading edge (104). The directional re-localization of the Golgi/centrosome has been attributed to the activation of Cdc42 at the leading edge which results in the recruitment of the Par6/aPKCz complex and the activation of aPKCz along microtubules. The membrane protrusion effect has been attributed to the Pak-Cool complex. The localization of the Pak-Cool complex to the

leading edge was suggested to restrict the Rac-mediated polarized membrane protrusions to the leading edge. This localization of the Cool-Pak complex and the localized activation of Cdc42 both involve activation of Cool which, in turn, has been shown to be under the regulation of Arf6 activity (106). Given that Cat has been already been shown to mediate the effects of Arf6 on cell protrusion, it is very likely that Cat is involved in this role of Cdc42 in cell polarity. In fact, Cat-2 has been shown to be a crucial protein for directional migration in wound-healing assays, strengthening the idea that Cat is involved in establishing the polarization of membrane protrusion in cells.

***GPCR and other receptor internalization***

When Cat was first identified as a GRK-binding partner by the Lefkowitz laboratory, the functional consequence of these interactions was shown to be linked to GPCR internalization (43). Lefkowitz and colleagues had noticed that the over-expression of Cat in cells led to the enhanced phosphorylation of GPCR by GRK as a result of retarded internalization of the receptor from the plasma membrane. However, expression of a mutant form of Cat that lacked this GAP domain did not lead to the build up of GPCRs on the cell surface, suggesting that Cat's GAP activity is important for mediating this outcome. More specifically, it was hypothesized that Cat's GAP activity toward the Arf6 GTPase leads to the inactivation of Arf6 which, in turn, blocked the internalization of GPCRs. The study was expanded to multiple endocytic pathways utilized by various receptors after stimulation. By detecting cell surface receptors by receptor-specific antibodies, it was concluded that the endocytic pathways that were affected by the over-expression of Cat were the same pathways that were under the control of clathrin-

mediated endocytosis, as well as being under the regulation of dynamin, and beta-arrestin (54).

**Overview of thesis**

Here I attempt to understand Cat's potential role in cellular transformation. Despite the fact that various binding partners of Cat have been shown to be involved in diverse signaling pathways and cellular functions, Cat is mostly known for its ability to influence cell morphology and migration. However, I felt that there were good reasons to examine the potential involvement of Cat in cellular transformation and growth. One such reason is that Cat is a major binding partner for Cool-1 which has already been shown to be a critical mediator of Cdc42 F28L-mediated transformation, as well as vSrc-dependent transformation. In chapter 2 of this thesis, I show that Cat has an important role in the anchorage-independent growth of both Cdc42 F28L-mediated transformed cells, and cervical carcinoma cell line, HeLa. Moreover, my results suggest that Cat acts as an effector for activated Arf GTPases in promoting transformation. Critical step in the effector function of Cat involves its ability to bind to paxillin. In chapter 3, I then provide evidence that paxillin is, to our surprise, is a negative regulator of the anchorage-independent growth. This is suggests that the critical role of Cat in promoting cellular transformation is inhibiting the negative impact of paxillin on the aberrant growth of cancer cells by binding to paxillin.

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## Chapter 2

# The adaptor protein and Arf GTPase-activating protein Cat-1/Git-1 is required for cellular transformation


## Abstract

Cat-1/Git-1 is a multi-functional protein that acts as a GAP (GTPase-activating protein) for Arf GTPases, as well as serves as a scaffold for a number of different signaling proteins. Cat-1 is best known for its role in regulating cell shape and promoting cell migration. However, whether Cat-1 might also contribute to cellular transformation is currently unknown. Here we show that ~95% of cervical tumor samples examined over-express Cat-1, suggesting that the up-regulation of Cat-1 expression is a frequent occurrence in this type of cancer. We further demonstrate that knocking-down Cat-1 from NIH3T3 fibroblasts expressing an activated form of Cdc42 (Cdc42 F28L), or from the human cervical carcinoma (HeLa) cell line, inhibits the ability of these cells to form colonies in soft agar, an *in vitro* measure of tumorgenicity. The requirement for Cat-1 when assaying the anchorage-independent growth of transformed fibroblasts and HeLa cells is dependent on its ability to bind paxillin, while being negatively impacted by its Arf-GAP activity. Moreover, the co-expression of Cat-1 and an activated form of Arf6 in fibroblasts was sufficient to induce their transformation. These findings highlight novel roles for Cat-1 and its interactions with the Arf GTPases and paxillin in oncogenic transformation.

**Introduction**

Cat (for Cool-associated tyrosine phosphorylated), also referred to as Git (for G-protein coupled receptor kinase interactor) or PKL (for paxillin kinase linker), is a member of a family of multi-functional proteins that are known to regulate the activity of certain members of the Arf (for ADP ribosylation factor) family of small GTPases, as well as function as signaling scaffolds via its numerous protein binding domains (1-3). These proteins were discovered more than a decade ago through three independent lines of investigation. In one such study performed by our laboratory (1), Cat-1 and Cat-2 were identified as phosphorylated proteins that bound to Cool-1 (Cloned-out-of-library)-1/beta-Pix (Pak-interacting exchange factor), a guanine nucleotide exchange factor (GEF) for the small GTPases Cdc42 and Rac. This occurred as part of a larger effort aimed at identifying proteins that could regulate the activity of the p21-activated kinases (Paks), which serve as effectors of the small GTPases Cdc42 and Rac (1). Although it was shown that the Cat proteins do not directly regulate Pak activity (1), it was later demonstrated that Cat-1 plays a role in regulating the cellular localization of Pak (4,5)

In a second line of investigation, Git-1 was identified in a yeast two-hybrid screen as a binding partner for members of a family of serine/threonine protein kinases, collectively referred to as Grks (for G protein-coupled receptor kinases), that phosphorylate activated G-protein coupled receptors (GPCRs) (6). This interaction was suggested to contribute to the desensitization and Arf-mediated internalization of GPCRs, through the ability of Git-1 to inactivate Arf GTPases by virtue of its Arf-GAP activity (6-8).

PKL was identified in yet another screen as a binding partner for paxillin, a scaffold protein that plays an important role in regulating the formation of focal adhesions (9). These dynamic cellular structures are composed of various proteins, including integrins, that physically attach cells to their extracellular matrix, as well as mediate signaling events that are triggered by cellular interactions with the extracellular environment. With the goal of better understanding the proteins that interact with paxillin to regulate focal complex dynamics, PKL was identified as a paxillin-binding partner via pull-down assays where the LD4 motif of paxillin was used as bait. It was subsequently shown that PKL was responsible for mediating an interaction that occurred between paxillin and Pak, an important step in focal complex regulation (4,10).

Since the Cat/Git/PKL proteins (from here on collectively referred to as Cat) are able to interact with a variety of signaling proteins, they have the potential to influence several different cellular functions. Indeed, Cat-1 has recently been implicated in stimulating neuronal synaptic activity (11), and dendritic spine formation (12,13), as well as in inducing T-cell activation (14). Moreover, Cat-1 has been implicated in attention deficit hyperactivity disorder and in Huntington's disease (15,16).

However, most studies to date have linked Cat to changes in cell morphology and migration (10, 17-23). The involvement of Cat in cell migration has stemmed largely from findings demonstrating that its over-expression in fibroblasts leads to the loss of focal complexes (18). Recently, our laboratory has shown that the tyrosine phosphorylation/de-phosphorylation cycle of the Cdc42 GEF, Cool-1, regulates the interaction between Cat-1 and paxillin (20). This, in turn, drives focal complex assembly and dis-assembly in Src-transformed NIH3T3 fibroblasts,

thus leading to the enhanced migration and invasive activity exhibited by these cells. Cat was also shown to regulate the spreading of cells by helping recruit Pak or Erk to sites of adhesion via its interaction with paxillin (10), Cool-1, and Mek (21,22). In addition, through its ability to function as a GAP and inactivate the small GTPase Arf6, Cat influences lipid trafficking, an outcome that also has important consequences in cell spreading (23).

Although Cat-1 has been implicated in the regulation of a number of cellular processes and outcomes, an interesting question concerns whether it might contribute to cancer progression and the aberrant growth exhibited by transformed cells. Here we show that this is indeed the case. We first demonstrate that knocking-down Cat-1 in NIH3T3 fibroblasts stably expressing an oncogenic form of Cdc42, Cdc42 F28L, blocks their ability to exhibit anchorage-independent growth. We then show that Cat-1 is over-expressed in a majority of human cervical carcinoma tumors and that knocking-down Cat-1 in a cervical carcinoma cell line (HeLa) blocks the transformed phenotypes exhibited by these cells. We further demonstrate that the ability of Cat-1 to function as an Arf-GAP, as well as to interact with paxillin, has important consequences for cellular transformation. Moreover, the ectopic co-expression of Cat-1, with an activated form of Arf6, is sufficient to induce cellular transformation in NIH3T3 fibroblasts. Collectively, our findings point to an interesting and previously unappreciated role for Cat-1 in transformation and cancer progression.

**Experimental Procedures**

*Reagents*- The Cat-1, Arf1 and Arf6 antibodies were purchased from Santa Cruz Biotechnology. The V5 antibody was from Invitrogen, the HA antibody was obtained from Covance, while the actin antibody was from Sigma.

*Plasmids*- The human Cat-1 cDNA and the human Arf1 and Arf6 cDNAs were cloned into the lentiviral vector pCDH-CMV-MOS-EF1-Puro (System BioSciences). The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to generate point mutants of Cat-1 that are defective in binding Cool-1 (D294K/E295R) (24), paxillin (K663E/K758E) (25), or are defective as an Arf-GAP (R39A) (26). Likewise, dominant-active forms of Arf1 (Q71L, T161A) (27,28) and Arf6 (D125N) (29), as well as dominant-negative Arf6 (T27N) (30), were also generated.

*Lentivirus Generation*- The various Cat-1, Arf1, and Arf6 constructs were transfected into HEK293T cells using Lipofectamine (Invitrogen). The mature lentiviruses shed into the culturing medium from the transfectants were collected and processed according to the manufacturer's instructions (Systems BioSciences, Mountain View, CA).

*Cell Culture*- HeLa cells were grown in RPMI 1640 medium containing 10% fetal bovine serum. Parental NIH3T3 mouse fibroblasts or NIH3T3 cells stably expressing a dominant-active form of Cdc42 (Cdc42 F28L) or Ras (Ras G12V) were grown in DMEM medium containing 10% calf serum. The siRNAs were introduced into cells using Lipofectamine 2000 (Invitrogen), whereas the wild-type and mutant constructs of Cat-1, Arf1 and Arf6 were introduced into cells via lentiviral infection. HeLa cells and NIH3T3 cells stably expressing V5-tagged pCDH vector alone, or various forms of V5-Cat-1, Arf1, and Arf6 were selected for and maintained by supplementing the appropriate growth medium with 0.175 μg/mL puromycin for HeLa cells, and 2 mg/mL puromycin for NIH3T3 cells stably expressing Cdc42 F28L.

*Immunoblot Analysis*- Cells were lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM b-glycerol phosphate, and 1 mM aprotinin). The lysates were resolved by SDS/PAGE, and then the proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by exposure to ECL reagent.

*Soft-Agar Assays*- HeLa cells, parental NIH3T3 cells, or NIH3T3 cells stably expressing Cdc42 F28L or Ras G12V transfected with various siRNAs or expression plasmids as indicated, were plated at a density of $5 \times 10^3$ cells/mL in medium containing 0.3% agarose onto underlays composed of growth medium containing 0.6% agarose in six-well dishes. The cultures were fed once a week, and after 14 days, the colonies were counted.

*Arf GTPase Activity Assays*- A glutathione-S-transferase (GST) fusion protein containing the N-terminal portion of Golgi-associated, gamma adaptin ear containing, Arf binding protein 3 (GGA3) pre-bound to a glutathione resin was purchased from Pierce. The activity assays were performed according to the manufacturer's instructions.

*Immunohistochemistry*- A human cervical carcinoma tissue array composed of normal cervical tissue and cervical tumor samples (U.S. Biomax, Inc., Rockville, MD) was deparaffinized in xylene and rehydrated using different concentrations of alcohol. The antigens were retrieved by heating the tissues in a sodium citrate buffer. After cooling, the tissues were blocked with 3% hydrogen peroxide solution, an avidin/biotin blocking solution (Vector Labs), and finally with horse serum (Vector Labs). The slides were then incubated with a 1:50 dilution of the Cat-1 antibody overnight. The primary antibody was then detected with the ABC staining kit (Vector

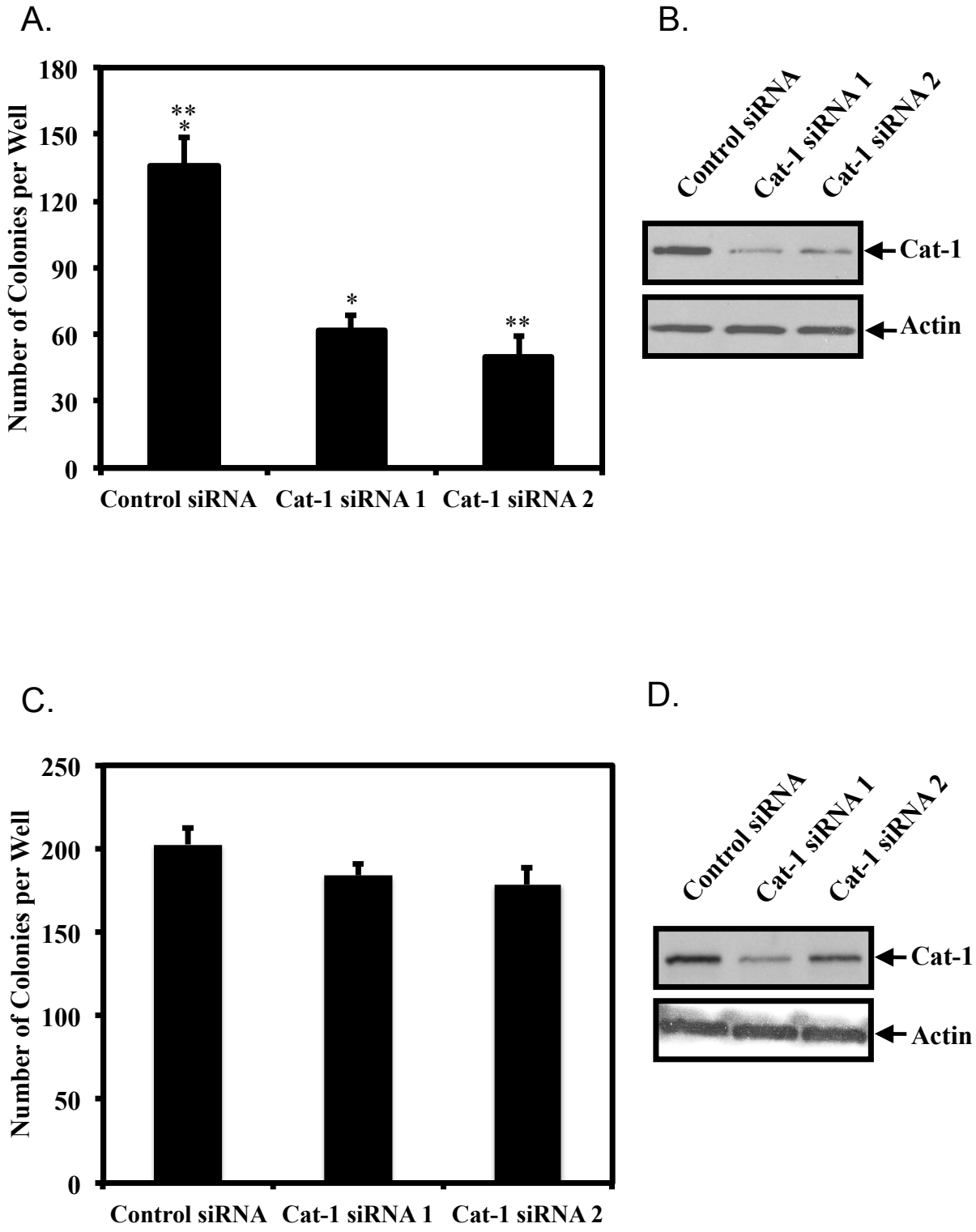
Labs) according to the manufacturer's manual. The resulting Cat-1 staining was analyzed with ImageJ.

*Immunoprecipitations*- Cell lysates (0.75~1 mg) that had been precleared with protein G beads were incubated with paxillin antibody for 1.5 h. Protein G beads were then added to the lysates and incubated for an additional 1 h, at which time the beads were washed extensively with cell lysis buffer. The resulting immunocomplexes were then subjected to Western blot analysis as indicated.

**Results**

To determine whether Cat-1 might contribute to oncogenic transformation, we examined whether it was required for the anchorage-independent growth exhibited by NIH3T3 fibroblasts stably expressing an oncogenic form of Cdc42, Cdc42 F28L (29). We began with Cdc42-transformed cells because we had earlier shown that Cool-1, a known Cat-1-binding partner, was essential for their transformation (31,32). Consistent with previous findings (29,33), NIH3T3 cells stably expressing the Cdc42 F28L mutant were capable of forming colonies in soft agar (Figure 2.1A, control siRNA). However, when Cat-1 expression was reduced in cells by at least 70% upon the introduction of either of two different Cat-1-specific siRNAs, there was a corresponding reduction in the number of colonies that were formed (Figures 2.1A and 1B). We have tried similar knock-down experiments in NIH3T3 cells stably expressing an oncogenic form of Ras (Ras G12V), although in these cases it has been difficult to achieve an equivalent reduction in Cat-1 expression. Nevertheless, we have found that under conditions where we obtained a 40 - 60% knock-down of Cat-1 expression, there was little effect on the ability of

**Figure 2.1 Cat-1 is necessary for the anchorage-independent growth of NIH3T3 fibroblasts expressing an oncogenic form of Cdc42, Cdc42 F28L.** *A and B,* Two sets of NIH3T3 cells stably expressing the Cdc42 F28L mutant were transfected with either a control siRNA or Cat-1 siRNAs (denoted as Cat-1 siRNA 1 or Cat-1 siRNA 2) as indicated. *A*, Soft-agar assays were performed on one set of the cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. *B*, The second set of transfected cells was lysed and subjected to Western blot analysis with Cat-1 and actin antibodies. The histograms show mean ± standard deviation (s.d.). Student's *t*-tests between indicated populations are *P<0.01, **P<0.01. *C and D*, Two sets of NIH3T3 cells stably expressing the Ras G12V mutant were transfected with either a control siRNA or Cat-1 siRNAs. *C*, Soft-agar assays were performed on one set of the cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. *D*, The second set of transfected cells was lysed and subjected to Western blot analysis with Cat-1 and actin antibody.


A.
B.
C.
D.
Number of Colonies per Well
180
150
120
90
60
30
0
**
*
*
**
Control siRNA
Cat-1 siRNA 1
Cat-1 siRNA 2
Cat-1
Actin
250
200
150
100
50
0

these cells to form colonies (Figures 2.1C and 2.1D). This may suggest that Cat-1 is less important for the transforming actions of oncogenic Ras.

We next examined Cat-1 expression levels in a variety of different human cancer cell lines. Cat-1 is more highly expressed in different cancer cell lines, as well as in NIH3T3 cells transformed by the Cdc42 F28L mutant, or by oncogenic v-Src, compared to its expression in either normal NIH3T3 cells or the human fibroblast cell line Detroit 551 (Figure 2.2). Because HeLa cells express relatively high levels of Cat-1, we then asked whether it was over-expressed in cervical cancers. A human tissue array consisting of 20 normal cervical tissues, together with 80 cases of primary cervical carcinomas, was subjected to immunohistochemical analysis. Figure 2.3A shows that Cat-1 can be detected in the normal cervical tissues, but at relatively low levels. However, the Cat-1 levels detected in nearly all of the cervical tumors were increased compared to their normal tissue counterparts. In fact, over 95% of the tumors were found to significantly over-express Cat-1 (Figure 2.3B). It is also worth noting that a correlation exists between tumor grade and the highest levels of Cat-1 expression (Figure 2.3C).

We then asked whether the increased levels of Cat-1 observed in the cervical tumors were important for their oncogenic phenotypes by taking advantage of our finding that the human cervical carcinoma cell-line, HeLa, expresses relatively high levels of Cat-1 (Figure 2.2). Either control siRNA or two different Cat-1-specific siRNAs were introduced into HeLa cells, and then the abilities of the cells to grow under anchorage-independent conditions were compared. Figure 2.4A shows that the Cat-1 siRNAs were effective at knocking-down Cat-1 in these cells, reducing its expression by ~90%. The ability of the HeLa cells depleted of Cat-1 to form

**Figure 2.2 Cat-1 is highly expressed in different types of human cancer cells and in transformed fibroblasts.** Western blot analysis was performed to detect Cat-1 expression in multiple cancer cell lines, mouse fibroblasts that stably express the vector alone or oncogenic forms of Src (v-Src) and Cdc42 (Cdc42 F28L) and in a human fibroblast cell line Detroit 551. The blot was reprobed with an actin antibody to confirm equal loading.

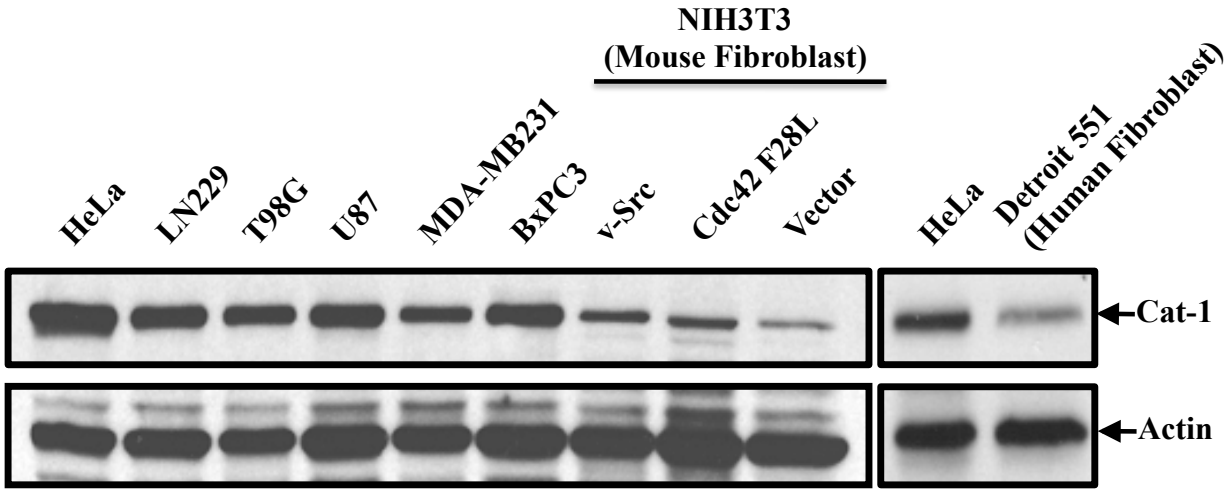
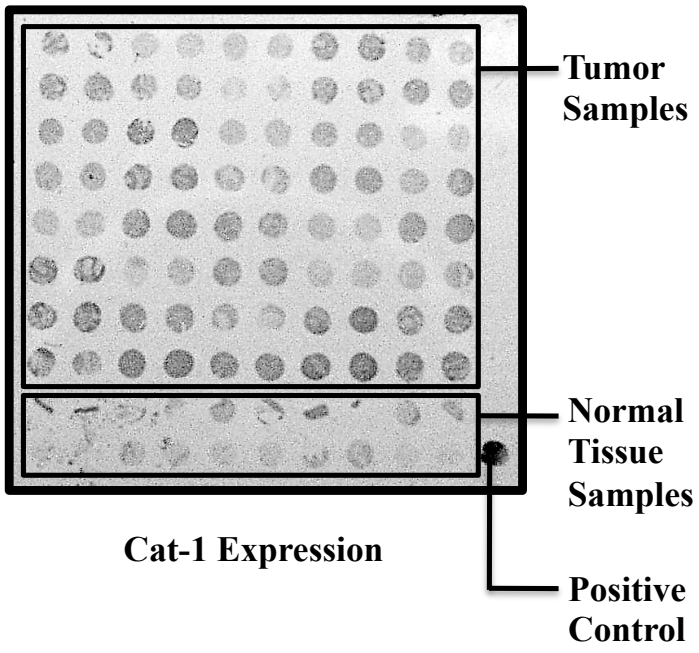
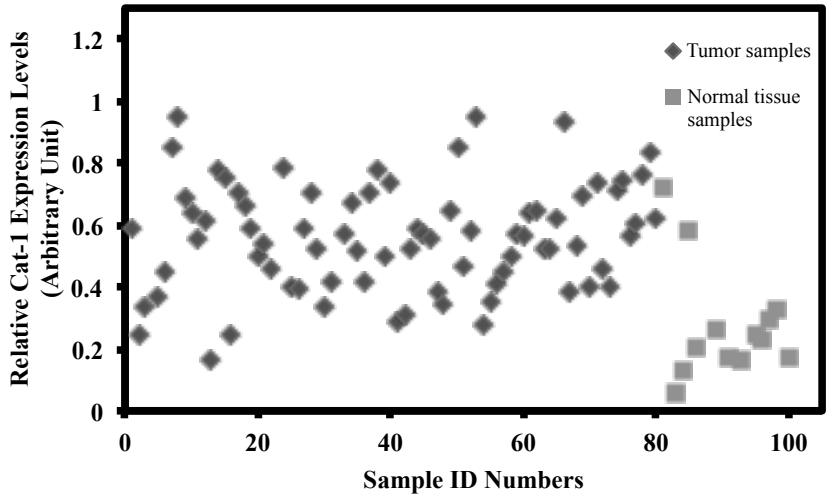

NIH3T3
(Mouse Fibroblast)
HeLa
LN229
T98G
U87
MDA-MB231
BxPC3
v-Src
Cdc42 F28L
Vector
HeLa
Detroit 551
(Human Fibroblast)
Cat-1
Actin

**Figure 2.3 Cat-1 expression is frequently enhanced in human cervical cancers.** *A*, A human cervical carcinoma tissue array consisting of 80 tumor samples and 20 normal tissues were subjected to immunohistochemical analysis using a Cat-1 antibody. *B*, The staining obtained with the Cat-1 antibody for each sample in the array was quantified using Image J and then plotted based on its relative expression of Cat-1. Each light colored square represents normal cervical tissue sample, while each dark diamond represents an individual cervical cancer case. *C*, The relative Cat-1 expression levels in the normal tissue and tumor samples were plotted based on tumor grade (from grade 1 to grade 3, as well as tumor samples whose grade was not available (N.D.)).

A.


Tumor Samples
Normal Tissue Samples
Positive Control
Cat-1 Expression

B.


Relative Cat-1 Expression Levels (Arbitrary Unit)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Sample ID Numbers
Tumor samples
Normal tissue samples

C.

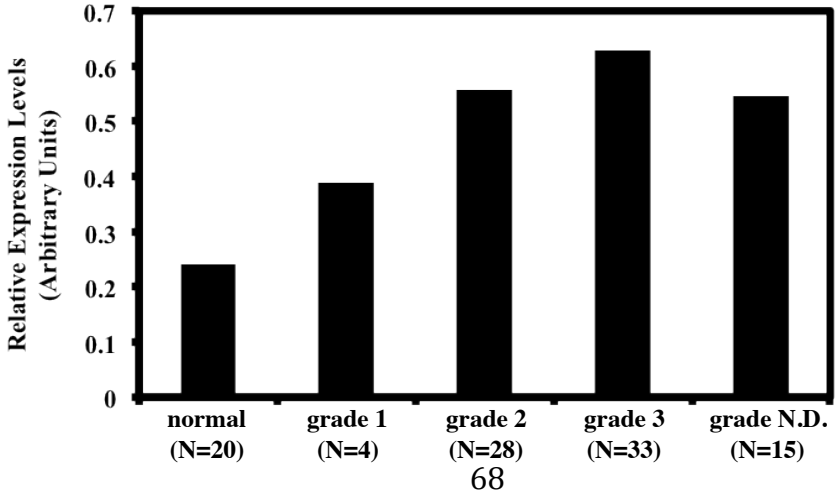
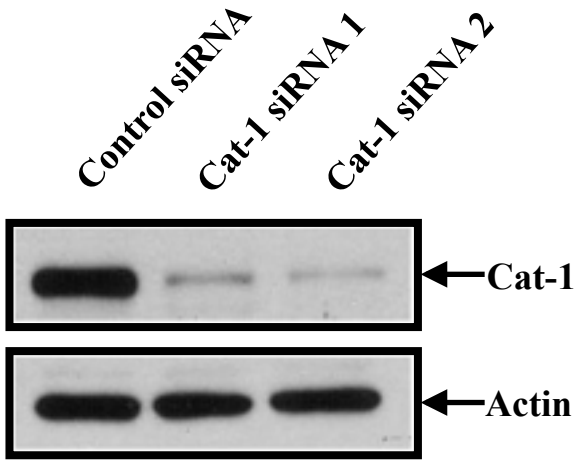
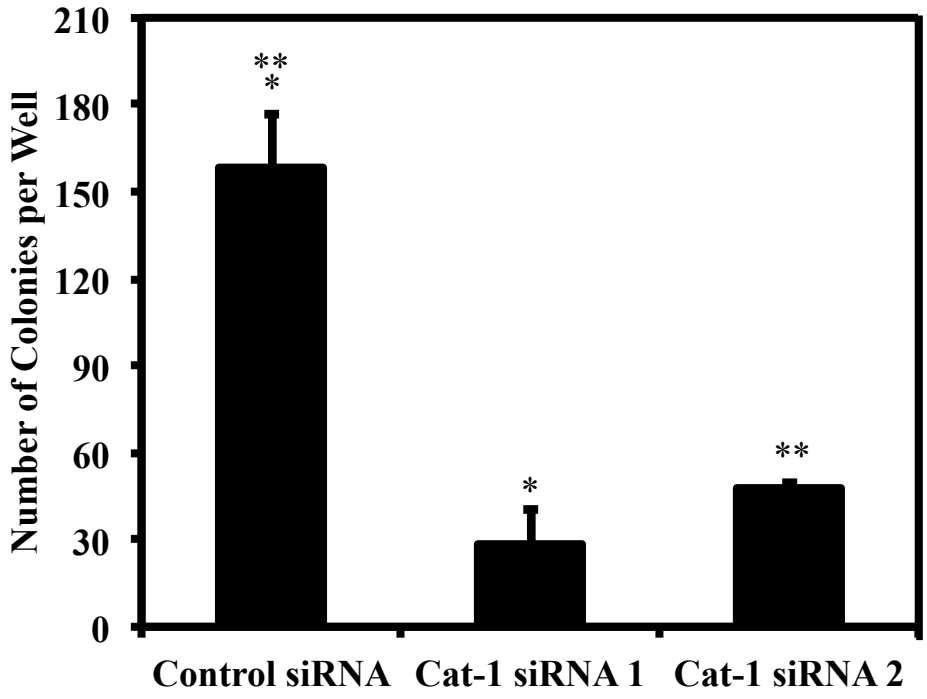

Relative Expression Levels (Arbitrary Units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
normal (N=20)
grade 1 (N=4)
grade 2 (N=28)
grade 3 (N=33)
grade N.D. (N=15)

**Figure 2.4 Cat-1 is necessary for the anchorage-independent growth of HeLa cervical carcinoma cells.** Two sets of HeLa cells were transfected with either a control siRNA or Cat-1 siRNAs (denoted as Cat-1 siRNA 1 or Cat-1 siRNA 2) as indicated. *A*, One set of the cells was lysed and then subjected to Western blot analysis with Cat-1 and actin antibodies. *B*, Soft-agar assays were performed on the second set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ± s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.01.

A.


Control siRNA
Cat-1 siRNA 1
Cat-1 siRNA 2
Cat-1
Actin

B.

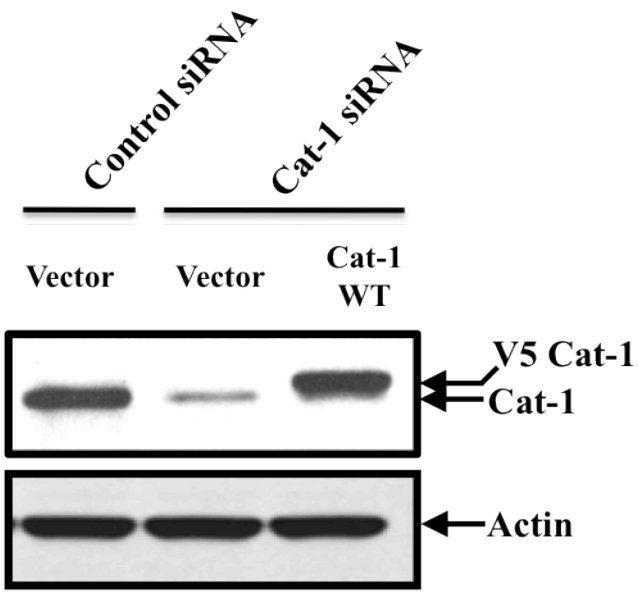

Number of Colonies per Well
210
180
150
120
90
60
30
0
**
*
*
**
Control siRNA
Cat-1 siRNA 1
Cat-1 siRNA 2

colonies in soft agar was significantly impaired compared to the control siRNA-treated cells, with at least 3-fold fewer colonies being formed by cells transfected with either of the Cat-1 siRNAs (Figure 2.4B). To rule out off-target effects of the Cat-1 siRNAs, a rescue experiment was performed where we attempted to restore the ability of HeLa cells transfected with a Cat-1 siRNA to exhibit anchorage-independent growth by re-introducing a V5-tagged, siRNA-insensitive form of Cat-1 via lentivirus infection. Figure 2.5A shows that following the introduction of this Cat-1 construct into HeLa cells depleted of endogenous Cat-1 (using Cat-1 siRNA 1), the expression of the V5-tagged Cat-1 was comparable to the endogenous levels of Cat-1 detected in the control HeLa cells (Figure 2.5A, compare lanes 1 and 3). As anticipated, restoring Cat-1 expression in these cells was able to completely rescue the growth inhibitory effects caused by the siRNA-targeted knock-downs (Figure 2.5B).

Having established that Cat-1 plays an important role in the anchorage-independent growth of transformed fibroblasts, as well as HeLa cervical carcinoma cells, we next examined which of the functions of Cat (i.e. its Arf-GAP activity, and/or its ability to associate with Cool-1 or paxillin) was responsible for mediating these effects. To address this question, we generated a series of V5-tagged Cat-1 mutant constructs that were insensitive to the effects of the Cat-1 siRNAs (Figure 2.6A). These various Cat-1 mutants were then introduced into HeLa cells that had been transfected with either a control siRNA or a Cat-1-specific siRNA. Western blot analysis was carried-out on the cells to determine the relative expression levels of endogenous Cat-1 in the control siRNA- and Cat-1 siRNA-treated cells, versus the levels of each of the ectopically expressed mutant forms of Cat-1 introduced into the Cat-1-depleted cells. Figure 2.6B shows that each of the siRNA-insensitive forms of Cat-1 expressed reasonably well in

**Figure 2.5 Re-introducing Cat-1 in Cat-knock-down HeLa cells restores their transforming ability.** HeLa cells stably expressing either the vector alone or an siRNA-insensitive form of wild-type Cat-1 were generated. Two sets of the vector alone-expressing cells were transfected with either control siRNA or Cat-1 siRNA and serve as the positive and negative controls in these experiments. The HeLa cells expressing the siRNA-insensitive Cat-1 WT were also transfected with the Cat-1 siRNA. *A*, One set of cells was lysed and subjected to immunoblot analysis using Cat-1 and actin antibodies. *B*, The second set of cells was subjected to soft-agar analysis and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ± s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.01.

A.


Control siRNA
Cat-1 siRNA
Vector
Vector
Cat-1 WT
V5 Cat-1
Cat-1
Actin

B.

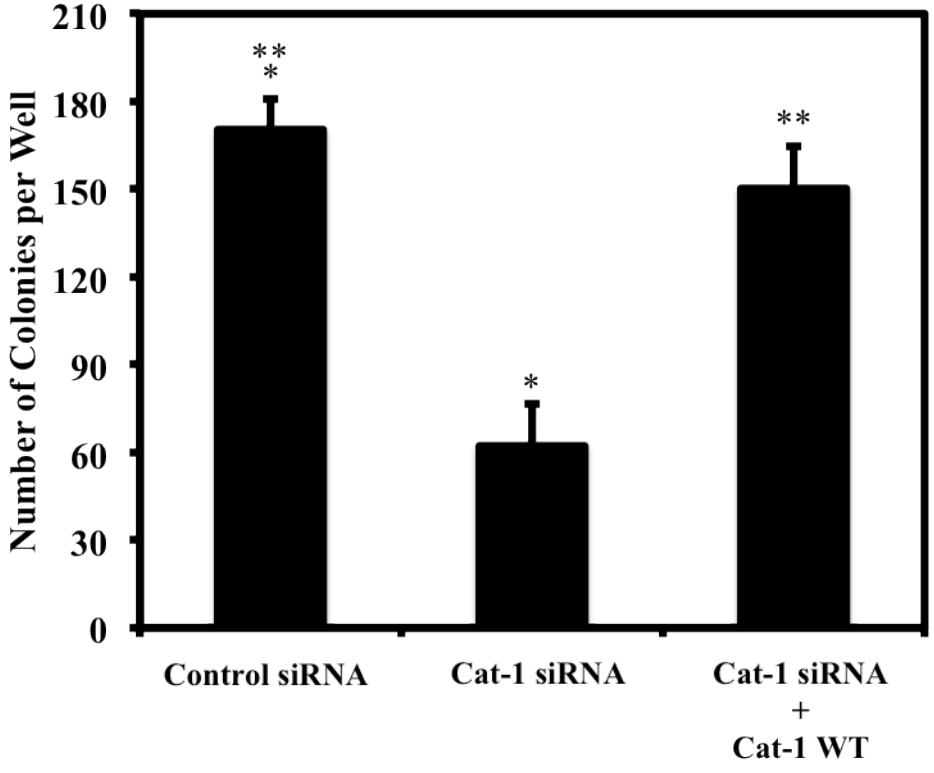
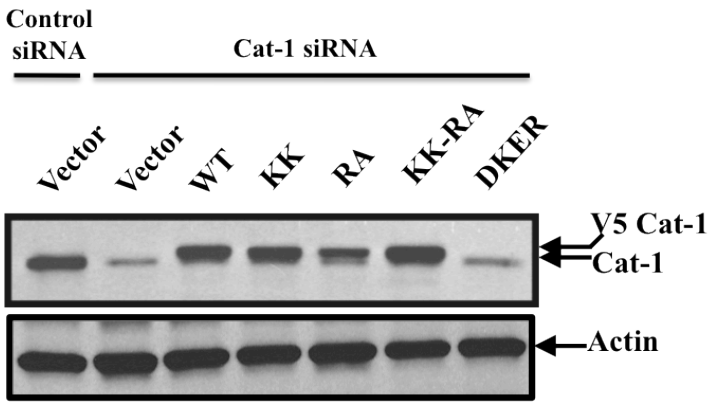

Number of Colonies per Well
210
180
150
120
90
60
30
0
**
*
*
**
Control siRNA
Cat-1 siRNA
Cat-1 siRNA + Cat-1 WT

**Figure 2.6 The ability of Cat-1 to interact with paxillin and function as an Arf-GAP is important for the anchorage-independent growth of HeLa cells.** *A*, List of the mutant forms of Cat-1 generated and the description of their functional defects. *B* and *C*, HeLa cells expressing either the vector alone or the various siRNA-insensitive mutant forms of Cat-1 were generated. Two sets of the vector alone-expressing cells were transfected with control siRNA or Cat-1 siRNA, and serve as the positive and negative controls in these experiments. HeLa cells expressing the various mutant forms of siRNA-resistant Cat-1 were also treated with Cat-1 siRNA. Later, one set of cells was lysed and subjected to Western blot analysis as indicated (*B*), while the second set of cells was subjected to soft agar analysis (*C*). The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ± s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.05, ***P<0.01.

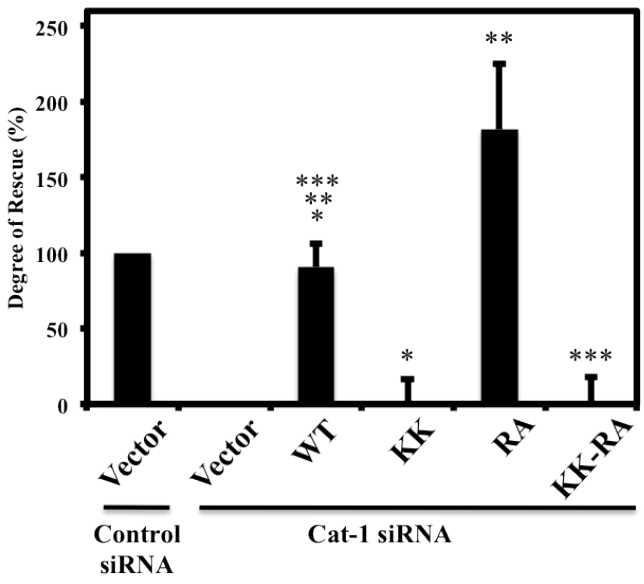
A.

| V5-tagged Cat-1 Constructs | Residue(s) Mutated | Functions Disrupted |
|---|---|---|
| WT | ---- | Wild type |
| KK | K663E/K758E | Pxn binding |
| RA | R39A | GAP activity |
| KK-RA | K663E/K758E & R39A | Pxn binding & GAP activity |
| DKER | D294K/E295R | Cool binding |

B.




C.



HeLa cells where endogenous Cat-1 expression had been knocked-down, with the exception of the Cat-1 mutant DKER that is defective in its ability to bind Cool-1 (Figure 2.6B, last lane). We tried several different titers of lentivirus as well as varied the amount of virus used to infect cells, but were never able to achieve the expression of this Cat-1 mutant. Consistent with our previous results, the introduction of a siRNA-insensitive form of wild-type (WT) Cat-1 into Cat-1-knock-down HeLa cells rescued the growth-inhibitory effects caused by depleting Cat-1 expression (Figure 2.6C, compare lanes 1, 2, and 3). However, cells expressing a mutant form of Cat that is defective in its ability to bind to paxillin (i.e. the KK Cat-1 mutant) could not restore the anchorage-independent growth of HeLa cells depleted of endogenous Cat-1 (Figure 2.6C). Interestingly, the form of Cat-1 defective for its Arf-GAP function (i.e. the RA mutant), not only rescued the growth-inhibitory effects of knocking-down Cat-1, but it consistently performed better than wild-type Cat-1 (Figure 2.6C, compare lanes 3 and 5). However, the enhanced colony formation stimulated by the Arf-GAP-defective form of Cat-1 was again dependent on its ability to bind to paxillin, since the Cat-1 double-mutant that is both incapable of functioning as an Arf-GAP and as a paxillin-binding partner (referred to as the Cat-1 KK-RA mutant) failed to promote anchorage-independent growth (Figure 2.6C, compare lanes 5 and 6). These data suggest that the role of Cat-1 in promoting cellular transformation is dependent on its ability to bind to paxillin and is consistent with the finding that ectopically expressing Cat-1 in HeLa cells leads to increased formation of Cat-1/paxillin complexes, as read-out by the co-immunoprecipitation of these proteins (Figure 2.7). Moreover, the enhanced transformation caused by the Arf-GAP-defective Cat-1 mutant implies that the activation of Arf GTPases could be important for malignant transformation.

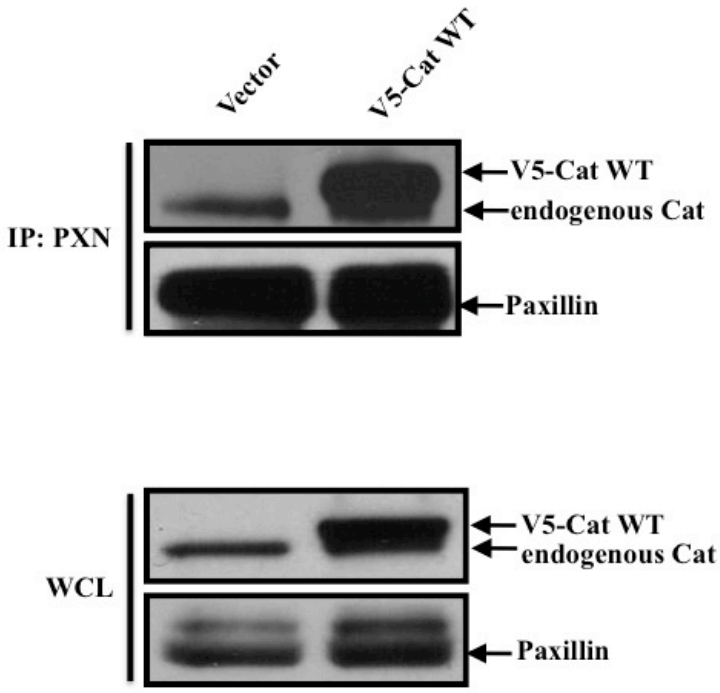
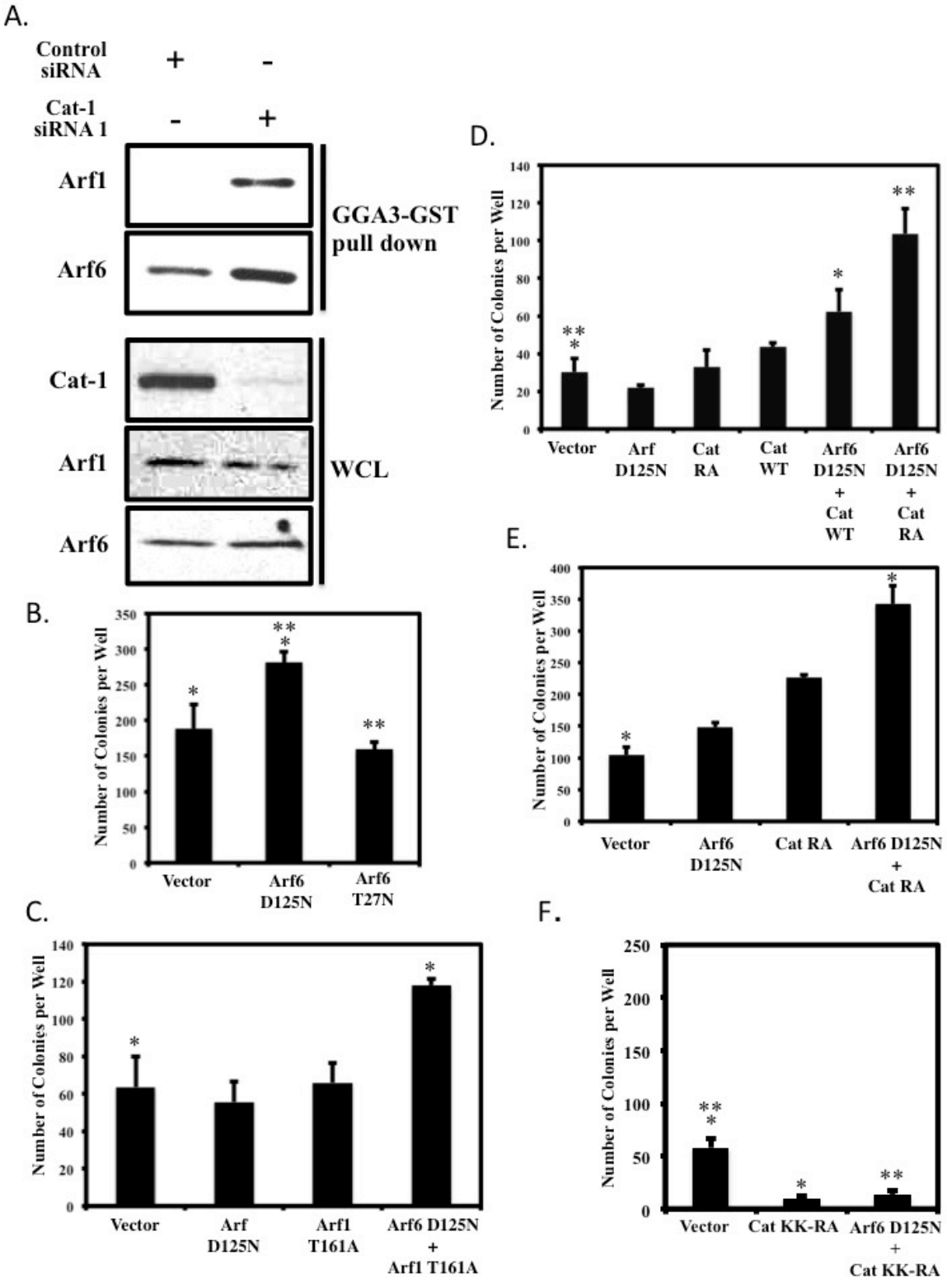



**Figure 2.7 Higher Cat expression leads to enhanced formation of Cat-paxillin complex.** Immunoprecipitations with a paxillin antibody were performed using extracts of HeLa cells ectopically expressing either vector alone, or V5-tagged Cat WT. The whole cells lysates (WCL) and the resulting immunocomplexes (IP:pxn) were immunoblotted with a Cat-1 and paxillin antibody.

We then set out to examine members of the Arf GTPase family that serve as substrates for the GAP activity of Cat-1 for their abilities to induce cellular transformation. We first asked whether the levels of activated, GTP-bound Arf1 or Arf6 were affected in HeLa cells under conditions where Cat-1 expression was knocked-down by RNAi. Although the Arf GTPase family is made-up of 6 members (Arf1-6), Arf1 and Arf6 have been shown to serve as substrates for Cat's GAP activity in cells (34,35). Therefore, we compared the relative levels of Arf1 and Arf6 activation in cells transfected with either a control siRNA or a Cat-1-specific siRNA, using GGA3-GST to pull down activated, GTP-bound Arf1 or Arf6 from cell lysates (36). Figure 2.8A shows that in HeLa cells expressing control siRNA, very little activated Arf1 is detected, whereas there is a detectable level of activated Arf6. However, when Cat-1 expression was knocked-down, both the levels of Arf1 and Arf6 activation were increased.

What then are the roles of these Arf GTPases in cellular transformation? To address this question, we generated a dominant-active mutant of Arf6, Arf6 D125N, capable of accelerated intrinsic nucleotide exchange (i.e. a "fast-cycling" mutant) based on the corresponding mutant for Cdc42 that we had previously characterized (29). In fact, when this Arf6 mutant was expressed in HeLa cells, it enhanced their ability to undergo anchorage-independent growth, whereas the dominant-negative Arf6 mutant, Arf6 T27N (30), was ineffective (Figure 2.8B). Expression of the activated Arf6 (Arf6 D125N) in fibroblasts was not sufficient to induce their transformation (Figure 2.8C, lane 2). The same was true for the activated form of Arf1 (Arf1 T161A), which was generated based on a previously reported fast-cycling Arf6 mutant (28). However, interestingly, when the activated Arf1 and Arf6 mutants were co-expressed in NIH3T3 cells, colony formation in soft-agar was enhanced (Figure 2.8C, lane 4).

**Figure 2.8 Cat-1 and Arf GTPases work together to promote cellular transformation.** *A*, HeLa cells stably expressing the vector alone or siRNA-insensitive forms of Cat WT and Cat RA were transfected with control siRNA or Cat-1 siRNA as indicated. Extracts from the cells were prepared and subjected to pull-down assays using the GGA3-GST fusion protein as bait. The activated forms of Arf1 and Arf6 were then detected by subjecting the pull-down complexes to immunoblot analysis using Arf1 and Arf6 antibodies, respectively. *B*, HeLa cells and *C*, NIH3T3 cells stably expressing various combinations of the vector alone, or dominant-active or -negative forms of Arf1 and Arf6, as indicated, were subjected to soft-agar analysis. These experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ± s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.01 for *B,* and *P<0.01 for C. *D*, NIH3T3 cells and *E*, HeLa cells stably expressing various combinations of the vector alone, Cat-1, and a dominant-active form of Arf6, as indicated, were subjected to soft-agar analysis. These experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ±s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.01 for *D*, and *P<0.01 for *E*. *F*, NIH3T3 cells stably expressing various combinations of the vector alone, Cat KK-RA, and a dominant-active form of Arf6, as indicated. These experiments were performed 3 times, and the results from each experiment were averaged together and graphed. The histograms show mean ±s.d. Student's *t*-tests between indicated populations are *P<0.01, **P<0.01.


A.
Control siRNA
+
-
Cat-1 siRNA 1
-
+
Arf1
Arf6
GGA3-GST pull down
Cat-1
Arf1
Arf6
WCL
B.
Number of Colonies per Well
Vector
Arf6 D125N
Arf6 T27N
C.
Number of Colonies per Well
Vector
Arf D125N
Arf1 T161A
Arf6 D125N + Arf1 T161A
D.
Number of Colonies per Well
Vector
Arf D125N
Cat RA
Cat WT
Arf6 D125N + Cat WT
Arf6 D125N + Cat RA
E.
Number of Colonies per Well
Vector
Arf6 D125N
Cat RA
Arf6 D125N + Cat RA
F.
Number of Colonies per Well
Vector
Cat KK-RA
Arf6 D125N + Cat KK-RA

The rescue experiments from Figure 2.6C suggested not only a novel role for activated Arf GTPases in transformation, but they also implied that this function may be dependent upon the binding of Cat-1 to paxillin. We therefore asked if the co-expression of Cat-1 with an Arf GTPase could promote cellular transformation. Figure 2.8D shows that expressing either activated Arf6 (D125N) alone in NIH3T3 cells, or Cat-1 alone, did not increase colony formation relative to control NIH3T3 cells (i.e., vector alone). However, co-expressing wild-type Cat-1 and the activated Arf6 (D125N) mutant gave rise to increased anchorage-independent growth of NIH3T3 cells, whereas co-expression of the Arf-GAP-defective Cat-1 mutant, Cat RA, and the activated Arf6 D125N mutant, even further enhanced colony formation. Similar results were obtained in HeLa cells (Figure 2.8E). The enhanced transformation that occurs in cells co-expressing the GAP-defective Cat-1 mutant together with Arf6 D125N was again shown to be dependent on the ability of Cat-1 to bind paxillin. Specifically, the co-expression of a mutant form of Cat-1 that cannot function as an Arf-GAP and is also defective in binding paxillin (Cat KK-RA), together with Arf6 D125N, was unable to induce anchorage-independent growth (Figure 2.8F).

**Discussion**

Cat-1 is a multi-functional protein that acts as a GAP for the Arf family of small GTPases and serves as a scaffold that interacts with various signaling proteins, perhaps most notably paxillin and Cool-1 (1-3,6,9). It has been implicated in a diverse range of cellular activities and outcomes, although it has been most often linked to cell morphology and promoting cell migration (10-13,17-23). To the best of our knowledge there has thus far not been any direct evidence demonstrating a role for Cat-1 in cell-growth control or in promoting oncogenic

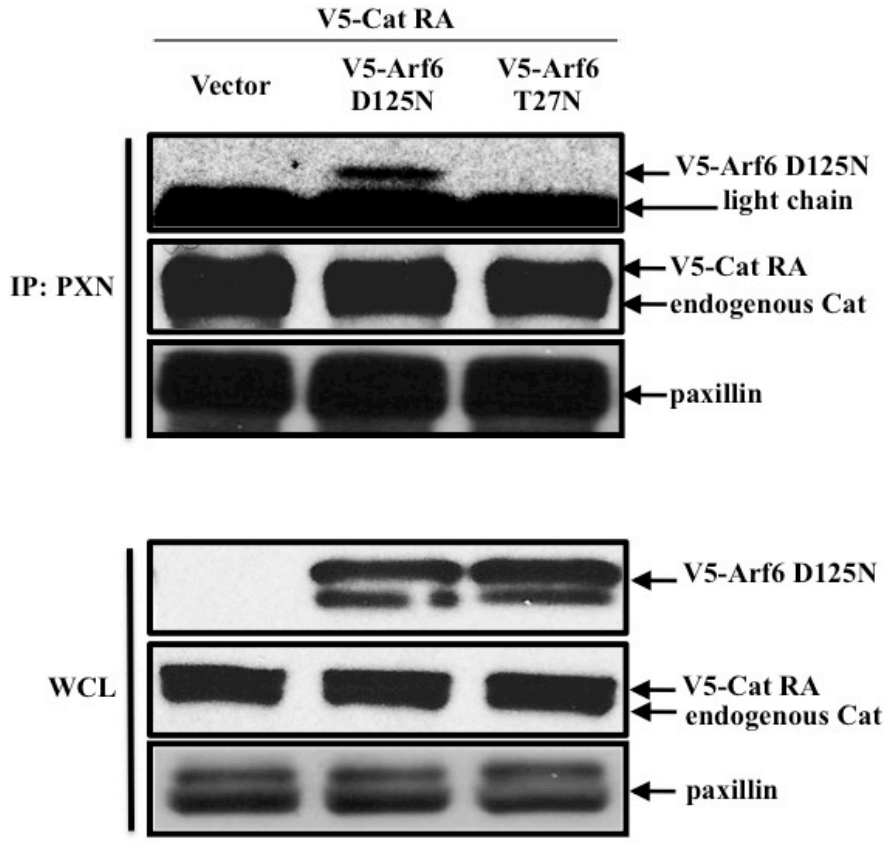
transformation. Here, we establish such a role for Cat-1 by showing that it is essential for anchorage-independent growth, an *in vitro* measure of tumorigenicity, exhibited by NIH3T3 mouse fibroblasts expressing a dominant-active form of the small GTPase Cdc42, as well as by the human cervical carcinoma cell line, HeLa. Moreover, we show that a vast majority of human cervical cancers over-express Cat-1, raising the intriguing possibility that it contributes to their progression.

These findings raise some interesting questions regarding how Cat-1 influences the growth of transformed cells. While the underlying mechanisms are likely to be complicated and involve a number of different proteins, our findings highlight two functions of Cat-1 that distinctly impact cellular transformation. On the one hand, they point to the ability of Cat-1 to interact with paxillin, another protein scaffold that binds various signaling proteins, as being an important step for anchorage-independent growth. This was highlighted by the fact that the mutant form of Cat-1 that is defective in its ability to bind paxillin fails to rescue the block on transformation caused by knocking-down endogenous Cat-1 in the HeLa cervical carcinoma cells, whereas the expression of a construct that encodes wild-type Cat-1 and is resistant to RNAi restores transformation. Paxillin has been shown in some cases to be required for the growth of cancer cells and transformed fibroblasts (37,38). These studies together with our own lead to suggestion that the formation of a Cat-1-paxillin complex might recruit and/or activate a unique set of signaling proteins that stimulate the growth of transformed cells. We are currently investigating whether the binding of Cat-1 to paxillin might potentiate the activation of a specific mitogenic signaling pathway(s) that is important for mediating transformation.

Additionally, our findings point to a functional interaction between Arf GTPases and Cat-1 that results in enhanced cellular transformation. In particular, we found that activated forms of Arf GTPases can promote cellular transformation and this effect appears to be dependent on Cat-1 binding to paxillin, as well as being enhanced in cells expressing a GAP-defective Cat-1 mutant. These results are also consistent with our finding that the ectopic expression of a GAP-defective form of Cat-1 consistently performed better than wild-type Cat-1 in restoring anchorage-independent growth to HeLa cells which lacked endogenous Cat-1 because of RNAi-treatment. Overall, these findings lead us to suspect that in certain cellular contexts, Cat-1 can act as an effector for activated Arf GTPases, perhaps through its ability to bind to paxillin and form a signaling scaffold that recruits proteins required for transformation. Indeed, Arf6 has already been implicated in anchorage-dependent growth (39) and a role for Arf-GAP as an Arf-effector has been proposed (40). Moreover, we were able to co-immunoprecipitate an activated mutant of Arf6 (Arf6 D125N) with paxillin from cells in which we ectopically expressed this Arf6 mutant together with an Arf-GAP-defective mutant of Cat-1 (Figure 2.9). Such an effector function of Cat-1 would presumably persist until its Arf-GAP activity converts Arf-GTP back to Arf-GDP, leading to a dis-assembly of the signaling scaffold.

Our findings linking Cat-1 to cellular transformation using cell-based assays prompted us to consider whether Cat-1 expression was up-regulated in human cancers. Although we have only just begun this phase of our studies by comparing Cat-1 expression levels in human cervical cancers to their corresponding normal tissue counterparts, what was especially striking was the number of cervical cancers that over-expressed Cat-1. Nearly 95% of the tumor samples examined showed enhanced levels of Cat-1, whereas normal cervical tissue samples showed

**Figure 2.9 Activated form of Arf6 specifically forms complex with Cat and paxillin.** Immunoprecipitations with a paxillin antibody were performed using extracts of HeLa cells ectopically expressing a paxillin binding-defective and a Arf GAP-defective mutant form of Cat, together with either vector alone, V5-tagged Arf6 D125N, or V5-tagged Arf6 T27N. The whole cell lysates (WCL) and the resulting immunocomplexes (IP:pxn) were immunoblotted with Arf6 and Cat-1 antibodies.


V5-Cat RA
Vector
V5-Arf6 D125N
V5-Arf6 T27N
IP: PXN
V5-Arf6 D125N
light chain
V5-Cat RA
endogenous Cat
paxillin
WCL
V5-Arf6 D125N
V5-Cat RA
endogenous Cat
paxillin

little, if any, Cat-1 expression. These findings are in agreement with the role that we have identified for Cat-1 in cell culture and thereby raise intriguing possibilities regarding a fundamental involvement of this protein in other types of cancers.

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# Chapter 3

## Cat promotes cellular transformation by inhibiting the negative impact of paxillin on transformation


### Abstract

Cat is an ArfGAP and a scaffold that has multiple domains that mediate various protein interactions. Cat has recently been implicated as having a role promoting anchorage-independent growth of cervical cancer cell line, HeLa, and also, in NIH3T3 cells transformed by stable expression of activated form of Cdc42, Cdc42 F28L. A key function of Cat that mediated this role was binding to paxillin. This was shown by demonstrating that an siRNA-insensitive form of Cat carrying a mutation that inhibits paxillin-binding was unable to restore the anchorage-independent growth property of HeLa cells that had been transfected with Cat siRNA. It is thus logical to ask the role of paxillin in cellular transformation in order to understand how Cat contributes to the aberrant growth by binding to paxillin. Here we show that paxillin, and Hic-5, a close homolog of paxillin that also binds to Cat, act to negatively regulate the anchorage-independent growth of HeLa cervical carcinoma cell line. Also, we show that Cat binding to paxillin, but not Hic-5, is the critical function of Cat that is important for mediating Cat's role in promoting anchorage-independent growth. Together these findings suggested that Cat, by binding to paxillin, inhibits the role that paxillin has on limiting cellular transformation. Finally, in accordance with the results from the soft agar assay, we show through Western

blot assay that knocking-down paxillin together with Cat in HeLa cells largely reverses the effects on signaling pathways influenced by knocking-down Cat alone. Together, these findings suggest a novl role of paxillin in the anchorage-independent growth of HeLa cervical carcinoma, and also gives new insight into the molecular mechanisms underlying Cat-mediated cellular transformation.

**Introduction**

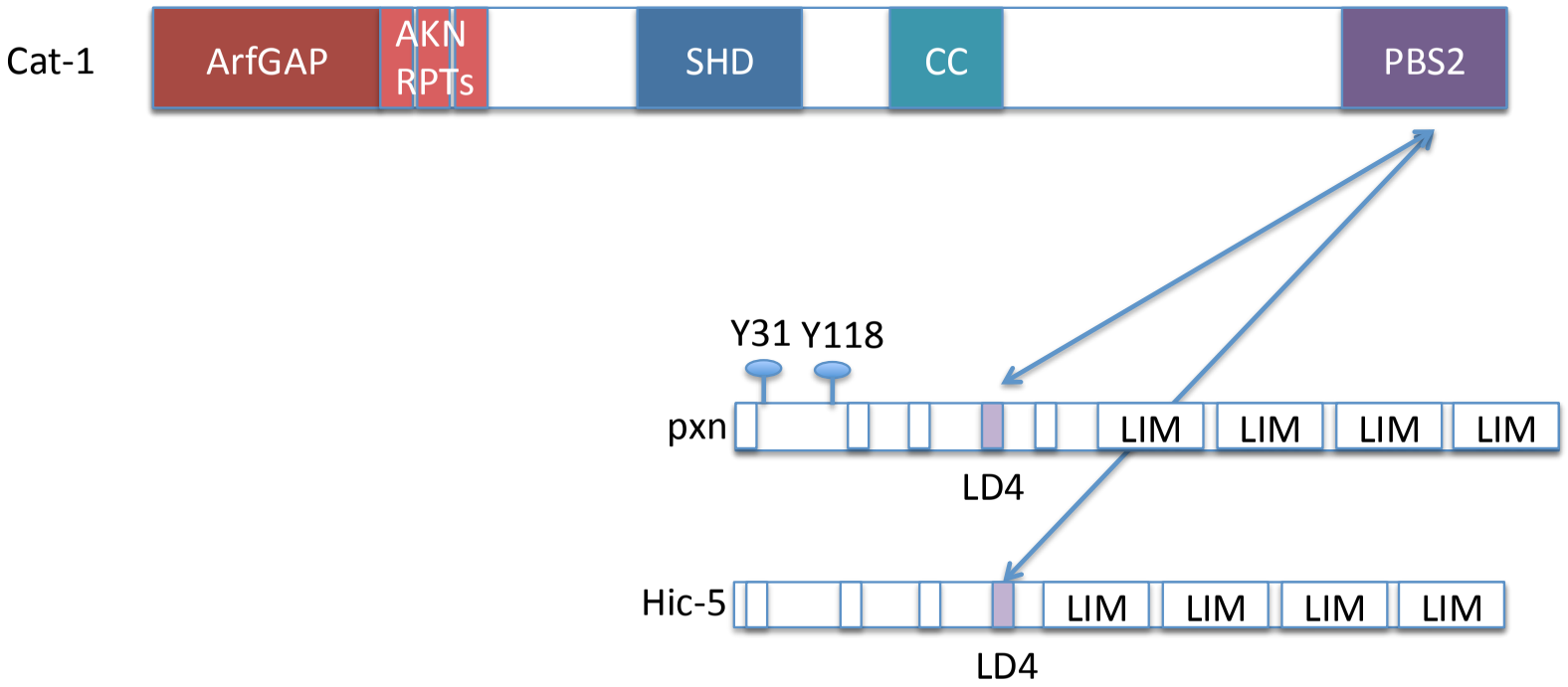
Anchorage-independent growth, or the ability of transformed cells to grow without being attached to the extracellular matrix, is a unique hallmark of cancer and transformed cells. Normal cells have a built-in system that leads to cell death, unless specific interactions occur between integrins and the extracellular matrix (ECM) proteins that make up the substratum (1). This mode of cell death is called anoikis, the Greek word for 'homelessness', and serves to limit cell growth to conditions only when the appropriate cell-ECM contacts are made. Given that the ability of cells to grow and survive without being attached to a substratum is thought to be a requirement for cancer cells to metastasize, understanding the mechanisms that underlie the ability of cancer cells to grow and survive under anchorage-independent conditions is of particular interest in cancer biology.

Cat is an Arf6 GAP and a scaffold protein that we have recently found to be over-expressed in a majority of human cervical carcinomas (S.Yoo et al., submitted). Moreover, knocking down Cat in HeLa cells, a human cervical carcinoma cell line, inhibited the ability of these cells to form colonies when grown under anchorage-

independent conditions. We then went on to show that Cat's ability to mediate this effect was dependent on its interaction with paxillin. This conclusion was based on the finding that a paxillin-binding-defective mutant of Cat, when introduced into cells lacking endogenous Cat (due to siRNA knock-down), was unable to restore the ability of these cells to form colonies whereas ectopically expressing a wild-type form of Cat was effective in restoring colony formation. In order to further understand how Cat and paxillin work together to promote tumorigenesis, we set out to better understand how paxillin influences cellular transformation.

Paxillin is an adapter protein that has been shown to have a major role in regulating focal adhesion dynamics and to participate in promoting integrin-mediated signaling events (2) (Figure 3.1). As was one of the first proteins to be identified as a constituent of focal complexes (3), paxillin was shown to accumulate at nascent focal complexes in migrating cells (4). It was also demonstrated through mutagenesis studies that disrupting the phosphorylation of paxillin by tyrosine kinases, such as the focal adhesion kinase (Fak), or by blocking paxillin's ability to interact with proteins like Cat or tubulin, alters focal complex dynamics and leads to irregular cell spreading and defects in cell migration (5,6). Interestingly, paxillin has also been reported to have a role in promoting the aberrant growth of transformed and human cancer cells. For example, paxillin was shown to be essential for the Ras-mediated transformation of fibroblasts (7). This role for paxillin was suggested to be related to its ability to promote the phosphorylation of Fak in suspension, which has previously been shown to have important implications in promoting anchorage-independent growth. In another study, it was shown that tyrosine

**Figure 3.1. A schematic showing the various domains of Cat-1, paxillin and Hic-5.** The diagram shows the domains used by Cat-1 and its binding partners, paxillin and Hic-5, to interact with each other. Tyrosine residues in paxillin that have previously been identified shown to be important for regulating cell migration are denoted.


Cat-1
ArfGAP
AKN RPTs
SHD
CC
PBS2
Y31 Y118
pxn
LIM
LIM
LIM
LIM
LD4
Hic-5
LIM
LIM
LIM
LIM
LD4

88 in paxillin was de-phosphorylated by protein tyrosine phosphatase receptor T (8). This phosphatase is one of the most frequently mutated tyrosine phosphatases in human cancer (9). Thus, in colon cancer cells lacking a functional protein tyrosine phosphatase receptor T, it was shown that phosphorylation of paxillin on tyrosine 88 was increased and this was important for the ability of the human colon cancer cell lines exhibit resistance to anoikis.

Interestingly, the point mutations within Cat that disable its binding to paxillin also impair Cat's ability to bind to another protein, hydrogen peroxide induced clone-5 (Hic-5), a closely related member of the paxillin family (10,11). Thus, it is possible that the interaction of Cat with paxillin, Hic-5, or with both of these proteins, is important for Cat's ability to promote the growth and transformed characteristics of HeLa cells.

Hic-5 was first identified as a gene whose expression was induced in response to the effects of transforming growth factor β 1 (TGFβ1) stimulation on the mouse osteoblastic cell line MC3T3 (12). The growth inhibitory effects of TGFβ1 were already well established by this time. Specifically, Nose and his colleagues had already demonstrated that the growth inhibitory effects of TGFβ1 were at least partially mediated through the up-regulation of hydrogen peroxide ($H_2O_2$) production (12,13). Subsequently they showed that Hic-5 gene expression is up-regulated in response to $H_2O_2$ (hence the name, hydrogen peroxide induced clone – 5) and that increases in the expression of Hic-5 were responsible for mediating the negative effects of $H_2O_2$ on cell proliferation. Subsequently, it was realized that Hic-5 shared close structural homology with paxillin through its

recurring LD motifs in its N-terminus and multiple LIM-domains at its C-terminus (Figure 3.1) (14). Moreover, the localization of Hic-5 in cells was similar to that of paxillin, being found at sights of focal adhesions, as well as in the nucleus. So far, the best-known cellular function of Hic-5 is its role in limiting cell growth and in promoting cell death (15-17). In the context of anchorage-independent growth, Hic-5 has recently been shown to be a major player in mediating the anchorage-dependent cell-cycle arrest of the mouse MC3T3 octeoblast cell line through a mechanism that involves its translocation from the cytoplasm to the nucleus when cells are detached from their substratum (18). Thus, the impact of paxillin and Hic-5 in cancer progression is just beginning to be appreiciated.

Here we have set out to characterize how Cat binding to paxillin and Hic-5 promotes the transformed characteristics exhibited by HeLa cervical carcinoma cells. Unexpectedly, we show that knocking-down either-paxillin or Hic-5 in HeLa cells enhances the ability of these cells to form colonies in soft-agar. In the case of paxillin, we confirmed its role in limiting the anchorage-independent growth of HeLa cells by showing that ectopic expression of paxillin in HeLa cells causes them to form less colonies compared to control cells that over-express the vector alone. Moreover, the growth inhibition caused by knocking-down Cat expression in HeLa cells can be overcome by knocking-down paxillin, but not Hic-5. Collectively, these results point to a new and unexpected role for the focal complex scaffold proteins, paxillin and Hic-5, as negative regulators of cellular transformation. Moreover, the data also suggests that Cat's function in promoting cellular

transformation is through its ability to bind paxillin, and thereby regulate their growth-inhibitory functions.

**Experimental Procedures**

*Reagents*- The Cat-1 antibody was purchased from Santa Cruz Biotechnology, paxillin antibody from Millipore. Hic-5, phospho-Erk, phospho-Fak, phospho-S6K, phosphor-Akt, phosphor-AMPK, Erk, Fak, S6K, Akt, AMPK antibodies were purchased from Cell Signaling. The V5 antibody was from Invitrogen, the HA antibody was obtained from Covance, while the actin antibody was from Sigma.

*Plasmids and siRNA*- The chicken paxillin-GFP constructs were from Chris Turner. The QuickChange™ site-directed mutagenesis kit (Stratagene) was used to generate point mutants of Cat-1 that are defective in binding paxillin (K663E/K758E) (25), and are defective as an Arf-GAP (R39A) (26). Paxillin-targeting and Hic-5-targeting siRNAs were purchased from Invitrogen.

*Cell Culture*- HeLa cells were grown in RPMI 1640 medium containing 10% fetal bovine serum. Parental NIH3T3 mouse fibroblasts or NIH3T3 cells stably expressing a dominant-active form of Cdc42 (Cdc42 F28L) were grown in DMEM medium containing 10% calf serum. The siRNAs were introduced into cells using Lipofectamine 2000 (Invitrogen), whereas plasmid transient transfection were introduced using Lipofectamine in combination with Plus reagent from Invitrogen.

*Immunoblot Analysis*- Cells were lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM b-glycerol phosphate, and 1 mM aprotinin). The lysates were resolved by SDS/PAGE, and then the

proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by exposure to ECL reagent.

*Soft-Agar Assays*- HeLa cells, parental NIH3T3 cells, or NIH3T3 cells stably expressing Cdc42 F28L transfected with various siRNAs or expression plasmids as indicated, were plated at a density of $5 \times 10^3$ cells/mL in medium containing 0.3% agarose onto underlays composed of growth medium containing 0.6% agarose in six-well dishes. The cultures were fed once a week, and after 14 days, the colonies were counted.

**Result**

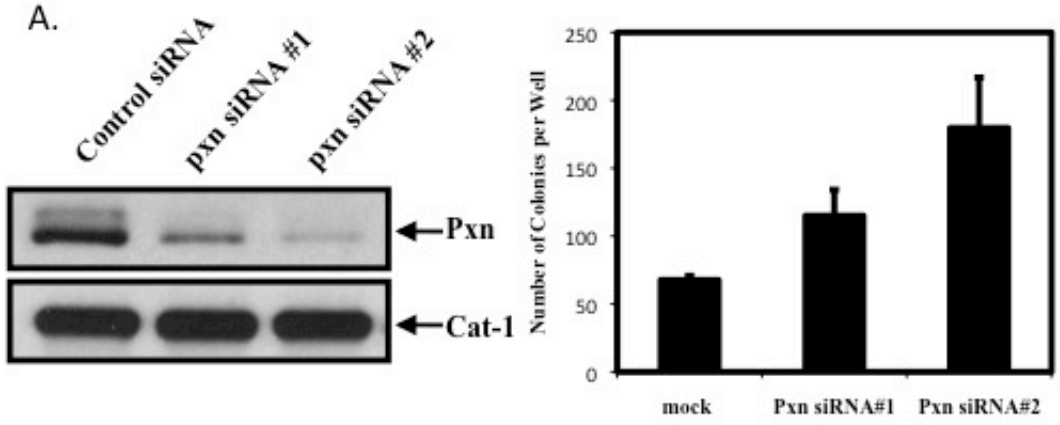
I have recently shown that Cat, an ArfGAP that contains multiple protein-protein interaction domains that mediate its scaffolding function, has an important role in promoting the transformed phenotypes of the human cervical carcinoma cell line, HeLa, as read-out by their ability to grow and form colonies in soft agar (Yoo et al., submitted). Moreover, I found that the ability of Cat to bind to paxillin, another scaffold protein that localizes to focal complexes (Figure 3.1) and regulates cell migration, was important for Cat's role in promoting the transformed properties of HeLa cells. Ectopically expressed forms of activated Arf GTPase were also shown to contribute to cellular transformation, but this was also found to be dependent on Cat's ability to bind paxillin. Collectively, these results suggested a model where Cat, acting as an effector for activated ArfGTPases (particularly Arf1 and Arf6), promotes cellular transformation by binding to paxillin.

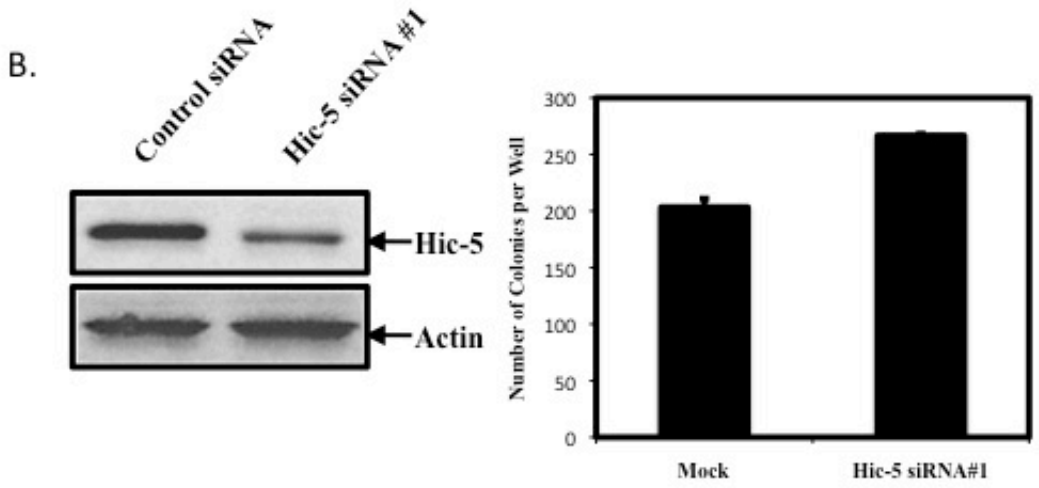
What, then, is the role of paxillin in promoting the anchorage-independent growth of HeLa cells? Paxillin has previously been shown to be important for the anchorage-independent growth of the colon cancer cell lines DLD1 and HCT116, as well as for fibroblasts stably expressing the oncogenic H-Ras G12V mutant (7,8). Moreover, paxillin was suggested to be critical for the mitogenic signaling initiated by EGF stimulation of the human prostate cancer cell lines LnCAP and PC3 (19). These findings initially led us to favor the idea that paxillin would have a positive role in promoting the transformed phenotypes of HeLa cells. However, when we assayed the ability to exhibit anchorage-independent growth following the transfection of two different siRNAs targeting paxillin (denoted as pxn siRNA#1, and pxn siRNA#2) (Figure 3.2A), we obtained a surprising result. Instead of finding that the knock-down of paxillin in these cells inhibited anchorage-independent growth, we discovered that reducing paxillin expression levels enhanced the ability of HeLa cells to form colonies in soft agar (Figure 3.2A). These findings then raised the possibility that Cat, by binding to paxillin, might prevent it from repressing cellular transformation.

The paxillin-binding defective form of Cat (designated 'KK' for K663E/K758E mutations), not only interferes with Cat's ability to interact with paxillin, but it also inhibits its binding to Hic-5, a close homolog of paxillin (Figure 3.1). Thus, we decided to examine whether knocking down Hic-5 in HeLa cells would impact cellular transformation. Either control siRNA or an siRNA targeting Hic-5 was introduced into HeLa cells, and then the abilities of the transfected cells to grow under anchorage-

**Figure 3.2 Paxillin and Hic-5 negatively affect the anchorage-independent growth of HeLa cervical carcinoma cells.** A. Two sets of HeLa cells were transfected with either a control siRNA or siRNA targeting paxillin (denoted as pxn siRNA#1 or pxn siRNA#2). One set of the cells was lysed and subjected to western blot analysis using paxillin and Cat antibodies. *(Top panel)*, Soft-agar assays were performed on the other set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times and the results from each experiment were averaged together and graphed. *(Bottom panel)*.

B. Two sets of HeLa cells were transfected with either a control siRNA or an siRNA targeting Hic-5 (denoted as Hic-5 siRNA#1). One set of cells was lysed and subjected to western blot analysis using paxillin and Cat antibodies. *(Top panel)*, Soft-agar assays were performed on the other set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times and the results from each experiment were averaged together and graphed. *(Bottom panel)*


A.
Control siRNA
pxn siRNA #1
pxn siRNA #2
Pxn
Cat-1
Number of Colonies per Well
250
200
150
100
50
0
mock
Pxn siRNA#1
Pxn siRNA#2


B.
Control siRNA
Hic-5 siRNA #1
Hic-5
Actin
Number of Colonies per Well
300
250
200
150
100
50
0
Mock
Hic-5 siRNA#1

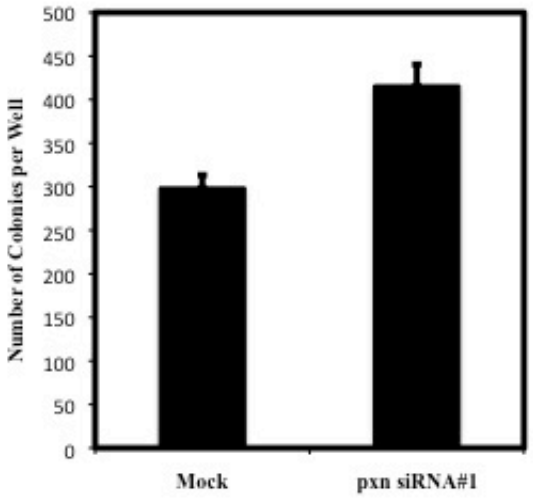
independent conditions were compared. Figure 3.2B shows that the Hic-5 siRNA was effective at knocking-down Hic-5, reducing its expression by ~60% compared to cells transfected with control siRNA. The same cells were then subjected to soft agar assays. Interestingly, knocking-down Hic-5 led to enhanced colony formation compared to control-siRNA treated cells, suggesting that both paxillin and Hic-5 are capable of negatively impacting the anchorage-independent growth of HeLa cervical carcinoma cells (Figure 3.2B).

We then asked if the negative effects that paxillin and Hic-5 have on cell growth also occur in NIH3T3 fibroblasts that constitutively express the oncogenic Cdc42 F28L mutant (Figure 3.3). We treated these cells with paxillin and Hic-5 siRNAs and then the cells were subjected to soft agar assays. Similar to the results we obtained in HeLa cells, Figure 3.3 shows that knocking-down either paxillin or Hic-5 in Cdc42 F28L-expressing NIH3T3 fibroblasts promoted colony formation in soft agar. Interestingly, simultaneously knocking-down both proteins in these cells did not enhance the extent of colony formation beyond what was seen when these proteins were individually knocked down, suggesting that paxillin and Hic-5 might use a similar mechanism to block cell growth.

To further confirm that paxillin and Hic-5 negatively impact cellular transformation, we next asked whether overexpressing these proteins in HeLa cells would inhibit their ability to grow in soft agar. Thus, we transiently over-expressed wild-type and several different mutant forms of paxillin in HeLa cells and subjected them to soft agar assays (Figure 3.4). One of the two paxillin mutants used lacks the LD4 motif that mediates the

**Figure 3.3 Negative effects of paxillin or Hic-5 on anchorage-independent growth are conserved in NIH3T3 cells stably expressing oncogenic form of Cdc42, Cdc42 F28L cells.** A. NIH3T3 cells stably expressing Cdc42 F28L were transfected with either a control siRNA or an siRNA targeting paxillin (denoted as pxn siRNA#1) as indicated. Soft-agar assays were performed and the resulting colonies that formed were counted. The experiments were performed 3 times and the results from each experiment were averaged together and graphed.
B. NIH3T3 cells stably expressing Cdc42 F28L were transfected with either a control siRNA, an siRNA targeting Hic-5 (denoted as Hic-5 siRNA#1), or an siRNA targeting paxillin (denoted pxn siRNA#1) as indicated. Soft-agar assays were performed on one set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed.

A.


Number of Colonies per Well
500
450
400
350
300
250
200
150
100
50
0
Mock
pxn siRNA#1

B.

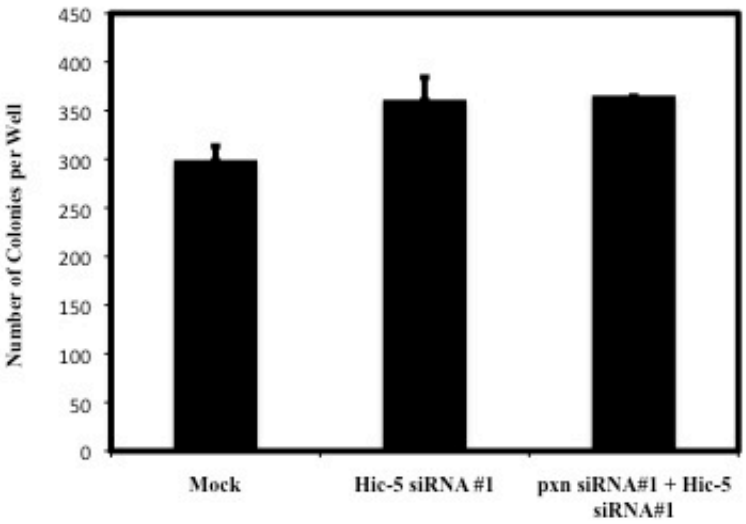
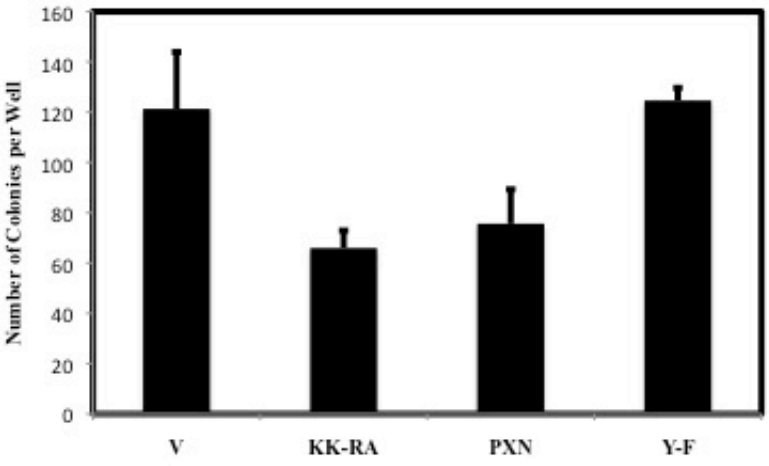

Number of Colonies per Well
450
400
350
300
250
200
150
100
50
0
Mock
Hic-5 siRNA #1
pxn siRNA#1 + Hic-5 siRNA#1

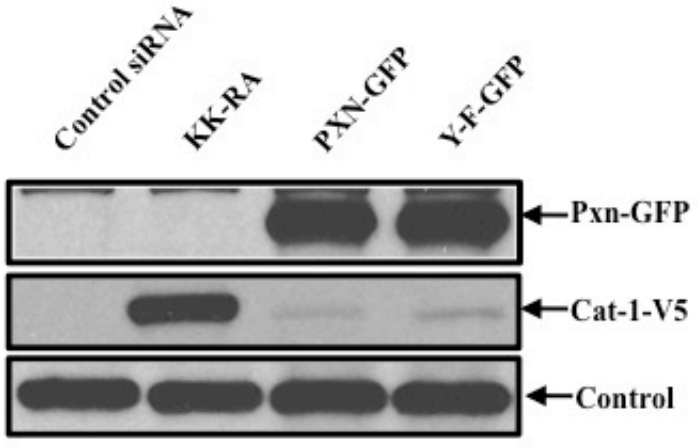
**Figure 3.4 Transiently over-expressing paxillin inhibits the anchorage-independent growth of HeLa cells.** *A*, List of the cDNA constructs encoding the various mutant forms of either Cat-1 or paxillin used in these studies, and the description of their functional defects. B. Two sets HeLa cells were either transiently transfected with vector alone or with various mutant forms of either Cat-1 or paxillin as indicated. *(Top panel)* Soft-agar assays were performed on one set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. *(Bottom panel)*, The second set of cells was lysed and then subjected to Western blot analysis with anti-V5, anti-GFP, and actin antibodies.

A.

| Ectopically Expressed Genes | Residues Mutated | Functions Disrupted |
|---|---|---|
| KK-RA | K663E/ K758E & R39A | Pxn binding & GAP |
| PXN | ---- | Wild type |
| Y-F | Y31F/ Y118F | Tyr to Phe |

B.



paxillin-Cat interaction (ΔLD4), while the other construct used has the two tyrosine residues (tyrosine 31, tyrosine 118) previously shown to be important for migration (2) mutated to phenylalanine. HeLa cells were also transfected with the vector only (as a positive control), or with a double mutant form of Cat (KK-RA) that is defective in binding to paxillin/Hic-5 and in functioning as an ArfGAP (as a negative control). Consistent with our earlier findings showing that knocking-down paxillin in HeLa cells promoted aberrant cell growth, transient over-expression of wild-type paxillin in HeLa cells led to ~50% fewer colonies compared to the vector-alone expressing cells. Importantly, the reduction in colony number observed in cells overexpressing paxillin is comparable to that in HeLa cells overexpressing the KK-RA mutant of Cat (the form of Cat that cannot bind paxillin or function as a GAP). Transient expression of the mutant form of paxillin that lacks the LD4 motif (ΔLD4) and therefore cannot bind to Cat, inhibited the anchorage-independent growth of HeLa cells to a similar degree as wild-type paxillin. However, cells that over-expressed the phosphorylation-defective form of paxillin had little effect on the ability of HeLa cells to form colonies in soft agar. These findings suggest that it is the phosphorylation of tyrosine 31 and tyrosine 118 in paxillin that is critical for the ability of paxillin to negatively influence cellular transformation.

Given that paxillin inhibits the anchorage-independent growth of HeLa cells as well as NIH3T3 cells expressing Cdc42 F28L, it is reasonable to suspect that Cat, by binding to paxillin, somehow prevents paxillin from inhibiting the growth of cancer/transformed cells. However, can the binding of Cat to paxillin and/or Hic-5 fully explain why knocking-down Cat inhibited the aberrant growth of HeLa cells or NIH3T3 cells

transformed by Cdc42 F28L? To address this question, we knocked-down both Cat and paxillin in HeLa cells and examined whether the cells could still form colonies in soft agar. We reasoned that if Cat's role in promoting cellular transformation is solely due to its ability to bind paxillin, then the simultaneous knock-down of paxillin and Cat should eliminate the growth inhibitory effects of knocking-down Cat alone. The same reasoning would also apply to Hic-5, and additionally, if the binding of Cat to both paxillin and Hic-5 were important, then knocking-down both paxillin and Hic-5 should allow cells depleted of Cat to regain the ability to form colonies. Figure 3.5 shows the results of these experiments when performed using HeLa cells. Whereas the knock-down of Hic-5 did not rescue the reduction of colony formation that occurred in Cat knock-down cells, knocking-down paxillin expression did restore this transformation phenotype. Knocking-down Hic-5, paxillin, and Cat resulted in nearly the same number of colonies as observed when just paxillin and Cat-1 were knocked-down.

Next, we questioned what signaling events might be responsible for mediating Cat's effects on cell growth (Figure 3.6). Interestingly, when cells transfected with mock siRNA or an siRNA targeting Cat were lysed and subjected to Western blot analysis, we noticed that the activities of several mitogenic signaling proteins, including Erk, Fak, and mTOR (represented by the S6K phosphorylation), were up-regulated in the Cat siRNA treated cells compared to the mock-siRNA-treated control cells (Figure 3.6A). The only exception to this trend was the PI3K pathway. (Interestingly, knocking-down paxillin often had the opposite effect of knocking-down Cat. For example, knocking-down paxillin resulted in less phosphorylation of Fak in suspended cells, compared to the levels

**Figure 3.5 Knocking-down paxillin is able to rescue the ability of HeLa cells depleted of Cat to form colonies in soft agar.** Two sets of HeLa cells were transfected with either control siRNA or with various combinations of siRNAs targeting Cat-1, paxillin, Hic-5 as indicated.
Soft-agar assays were performed on the one set of cells and the resulting colonies that formed were counted. The experiments were performed 3 times, and the results from each experiment were averaged together and graphed. *(graph)*, One set of cells was lysed and then subjected to Western blot analysis with Cat-1, paxillin, and Hic-5 antibodies (*bottom panels*).

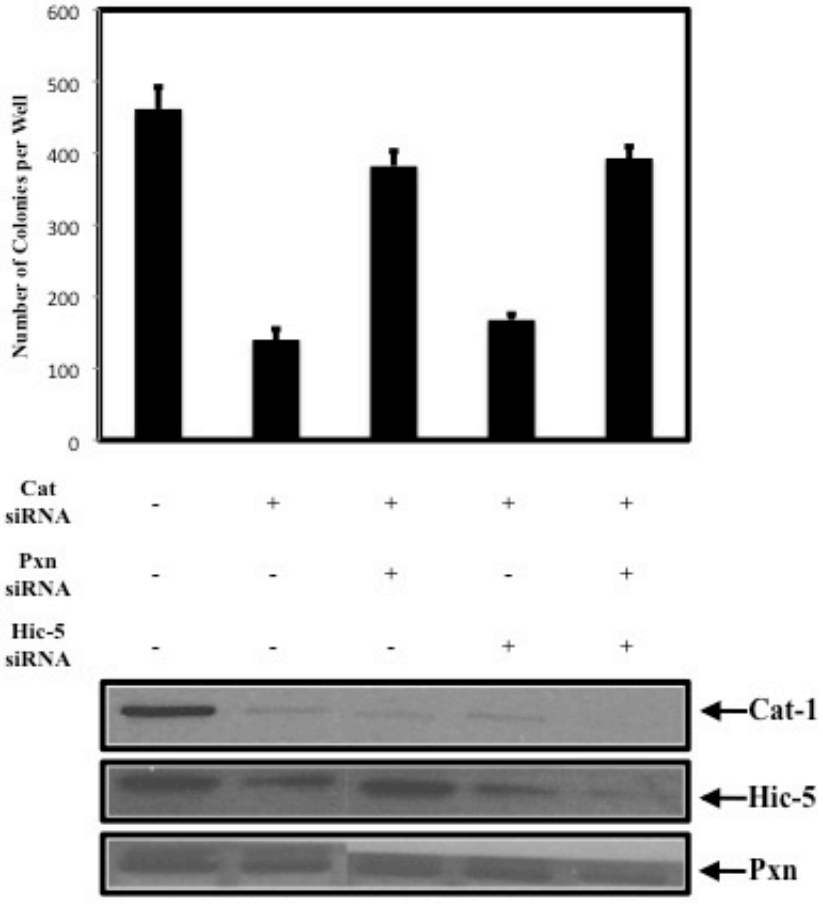
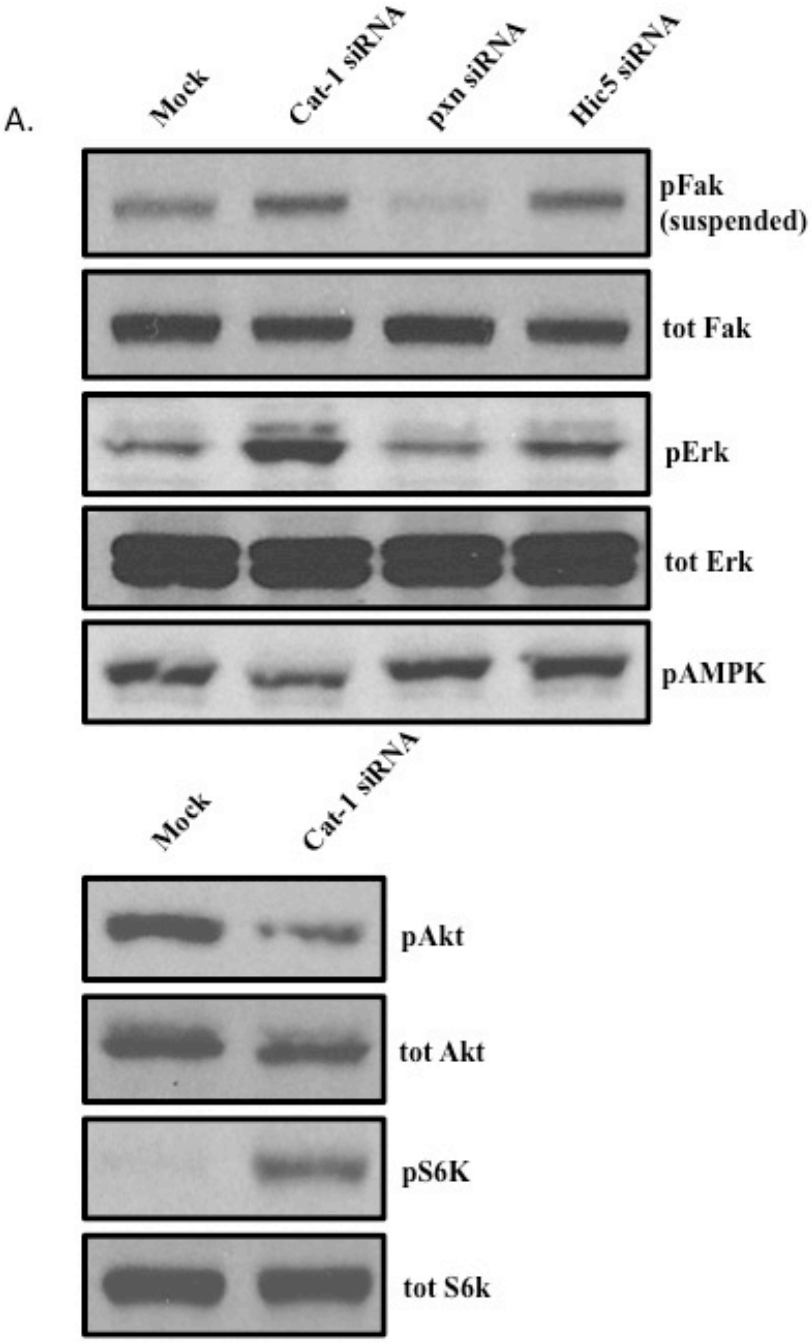
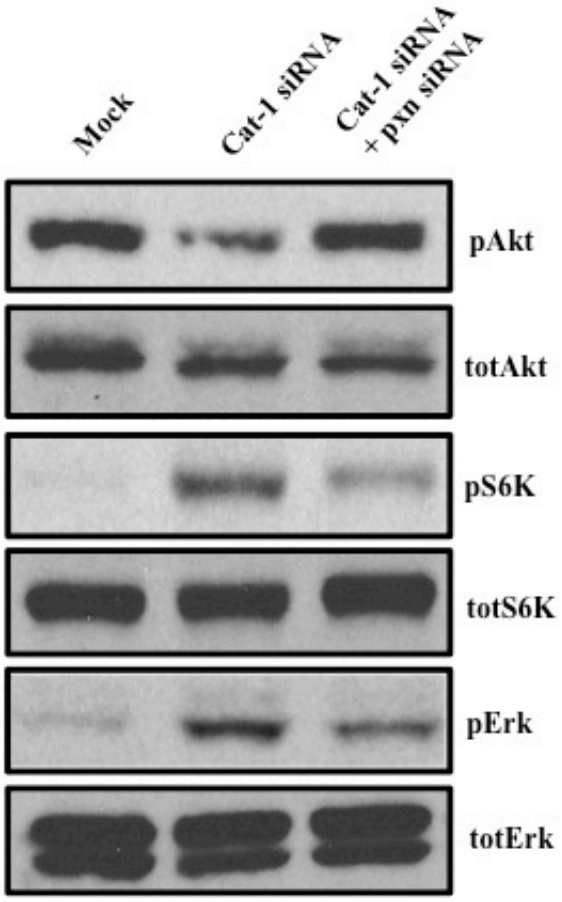

600
500
400
300
200
100
0
Number of Colonies per Well
Cat siRNA
Pxn siRNA
Hic-5 siRNA
- + + + +
- - + - +
- - - + +
Cat-1
Hic-5
Pxn

**Figure 3.6 Cat and paxillin-mediated effects on cell signaling.** A. Cultures of HeLa cells transfected with either mock, Cat-1, paxillin, Hic-5 siRNAs, and were grown under normal (10%FBS, RPMI) conditions for 48 hours and lysed. The whole cell lysates were immunoblotted with phospho-Fak, total-Fak, phospho-Erk, total-Erk, phospho-AMPK, total-AMPK, phospho-Akt, total-Akt, phospho-S6Kinase, total-S6Kinase antibodies. B. Cultures of HeLa cells transfected with either mock siRNA, or siRNAs targeting Cat-1 and paxillin were grown under normal (10%FBS, RPMI) conditions for 48 hours and then lysed. The whole cell lysates were immunoblotted with phospho-Akt, total-Akt, phospho-S6Kinase, total-S6Kinase, phospho-Erk, and total-Erk antibodies.


A.
Mock
Cat-1 siRNA
pxn siRNA
Hic5 siRNA
pFak
(suspended)
tot Fak
pErk
tot Erk
pAMPK
Mock
Cat-1 siRNA
pAkt
tot Akt
pS6K
tot S6k

B.


Mock
Cat-1 siRNA
Cat-1 siRNA + pxn siRNA
pAkt
totAkt
pS6K
totS6K
pErk
totErk

of phosphorylated Fak detected in mock-treated cells, whereas knocking-down Cat resulted in higher phosphorylation. This was in contrast with the results obtained when knocking-down Hic-5.) Also, consistent with the soft-agar result where knocking-down paxillin with Cat was enough to reverse the effects of knocking-down Cat alone, treating cells with both Cat and paxillin siRNAs reversed the signaling patterns that were observed when treating cells with Cat siRNA alone (Figure 3.6B).

**Discussion**

Cat is an ArfGAP that has recently been shown by our laboratory to be important for the anchorage-indepenent growth of HeLa cells as well as fibroblasts transformed by an oncogenic form of Cdc42 (Cdc42 F28L). We established a potential role for Cat in cancer progression by first showing that it was over-expressed in a majority of cervical cancers. Using colony formation in soft agar assays as an in-vitro measure of tumorigenicity, we then went on to demonstrate that the binding of Cat to paxillin was a key step in the role that Cat plays in transformation. Here, I expanded upon the findings by showing that a Cat-paxillin interaction seems to inhibit the negative effects that paxillin has on the anchorage-independent growth of HeLa cells. In doing so, these findings suggest a novel insight as to why Cat binding to paxillin/Hic-5 is important in cellular transformation.

While I had already shown that the binding of Cat to paxillin was in some way important for promoting the anchorage-independent growth of HeLa cells and fibroblasts expressing Cdc42-F28L, the underlying mechanism responsible for the actions of Cat was unclear. I set out to better understand the mechanism involved by seeing how

knocking-down paxillin and Hic-5 expression in HeLa cells influenced the ability of these cells to form colonies. Surprisingly, neither of these proteins was necessary for the transformed characteristics of HeLa cells. In fact, more colonies were formed by HeLa cells under conditions where the expression of paxillin or Hic-5 was depleted by siRNAs. This result was especially surprising for paxillin because it contradicts previous reports that have implicated roles for paxillin in the Ras-induced transformation of fibroblasts. However, Hic-5 has been implicated as a crucial mediator of the anchorage-dependent growth of normal cells, thus suggesting that it may be possible that knocking-down Hic-5 expression might actually potentiate the anchorage-independent growth of HeLa cells. In this context, I indeed observed that knocking-down Hic-5 in HeLa cells increased colony formation in soft agar. I also further confirmed the negative role for paxillin in cellular transformation by showing that its over-expression in HeLa cells reduced colony numbers. Moreover, I have identified that the phosphorylation of Tyr31 and Tyr118 to be important for the paxillin-mediated inhibition of the growth of HeLa cells. It is interesting to note that the two tyrosine residues have been previously shown to be necessary for binding the Crk adapter proteins, p85 PI3K and p120RasGAP (2). I am now setting out to determine whether the ability of paxillin to interact with any of these proteins mediates the negative effects that paxillin exerts on cell growth.

The fact that these focal complex proteins exerted negative effects on cellular transformation suggested a mechanistic model by which Cat binding to paxillin and/or Hic-5 might be important. Namely, Cat seems to have an antagonistic effect on the function of focal complex proteins through its interactions with these proteins. Recently

Hic-5 was shown to confer anchorage-dependence by accumulating in the nucleus in detached cells, which, in turn, prevented cyclin D from localizing to the nucleus, a critical step for cell cycle progression (18). Interestingly, paxillin has also been shown to traffic to the nucleus. Thus, it is attractive to consider that paxillin might be playing a similar role as Hic-5 in regulating transformation. Cat might, in turn, influence the nuclear localization of Hic-5, and potentially paxillin, by binding to these focal complex proteins and preventing their nuclear localization, thereby enabling cyclin D to localize to the nucleus and allow cell cycle progression to proceed.

This then raises an important question, namely, is the binding of Cat to paxillin and/or Hic-5 the only function of Cat necessary for its effects on cellular transformation? Or are there other functions of Cat that are also critical for its role in anchorage-independent growth? In the simplest sense, one might predict that if paxillin exerts a negative effect on cellular transformation, and that the binding of Cat to paxillin is the only critical function of Cat, then knocking-down paxillin together with Cat in HeLa cells should rescue the block in colony formation caused by knocking-down Cat alone. A similar argument applies for Hic-5. Interestingly, knocking-down just paxillin with Cat was able to rescue the block of colony formation by siRNA targeting Cat. This suggested that Cat binding to paxillin is sufficient to account for Cat mediated promotion of anchorage-independent growth. Indeed, we see that it is paxillin, but not Hic-5, that affects cell signaling to Cat when knocked-down in cells. In agreement with the soft agar data, knocking-down paxillin and Cat together can reverse the effects on signaling that were influenced by knocking-down Cat alone. In the near future, we hope to ask how paxillin

can negatively impact cell growth and what signals regulate Cat's inhibition of the negative regulatory functions of paxillin.

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# Chapter 4

## Conclusion

Understanding the molecular mechanisms that govern the growth and progression of human tumors serves as an important basis for the development of novel strategies to treat cancers. In this regard, little is known about Cat protein and its role in tumorigenesis. Cat is an Arf GAP protein that is also a scaffold that mediates the interactions between various proteins. In fact, Cat-1 was originally identified as a binding partner for a number of different proteins through pull-down assays. The Cerione laboratory used Cool-1, a GEF for Cdc42 and Rac GTPases, to pull-down Cat(1), whereas the Lefkowitz laboratory and Grk2(2), while the Turner laboratory used paxillin(3). The ability of Cat to interact with any of these proteins, suggests its potential involvement in various cellular functions. So far, however, the most-studied function of Cat is its role in cell morphology and focal complex regulation.

In my thesis work, I have investigated whether Cat might be involved in promoting cellular transformation. There were reasons to suspect such a role for Cat might exist, with perhaps the most notable of these having to do with its ability to interact with Cool-1. Cool-1 had already been shown to be a critical mediator of Cdc42-F28L-induced cellular transformation in fibroblast by not only functioning as a GEF for Cdc42 but also by serving as an effector of Cdc42(4). Since Cat-1 can bind to Cool, it made me wonder what role Cat might have in promoting cellular transformation.

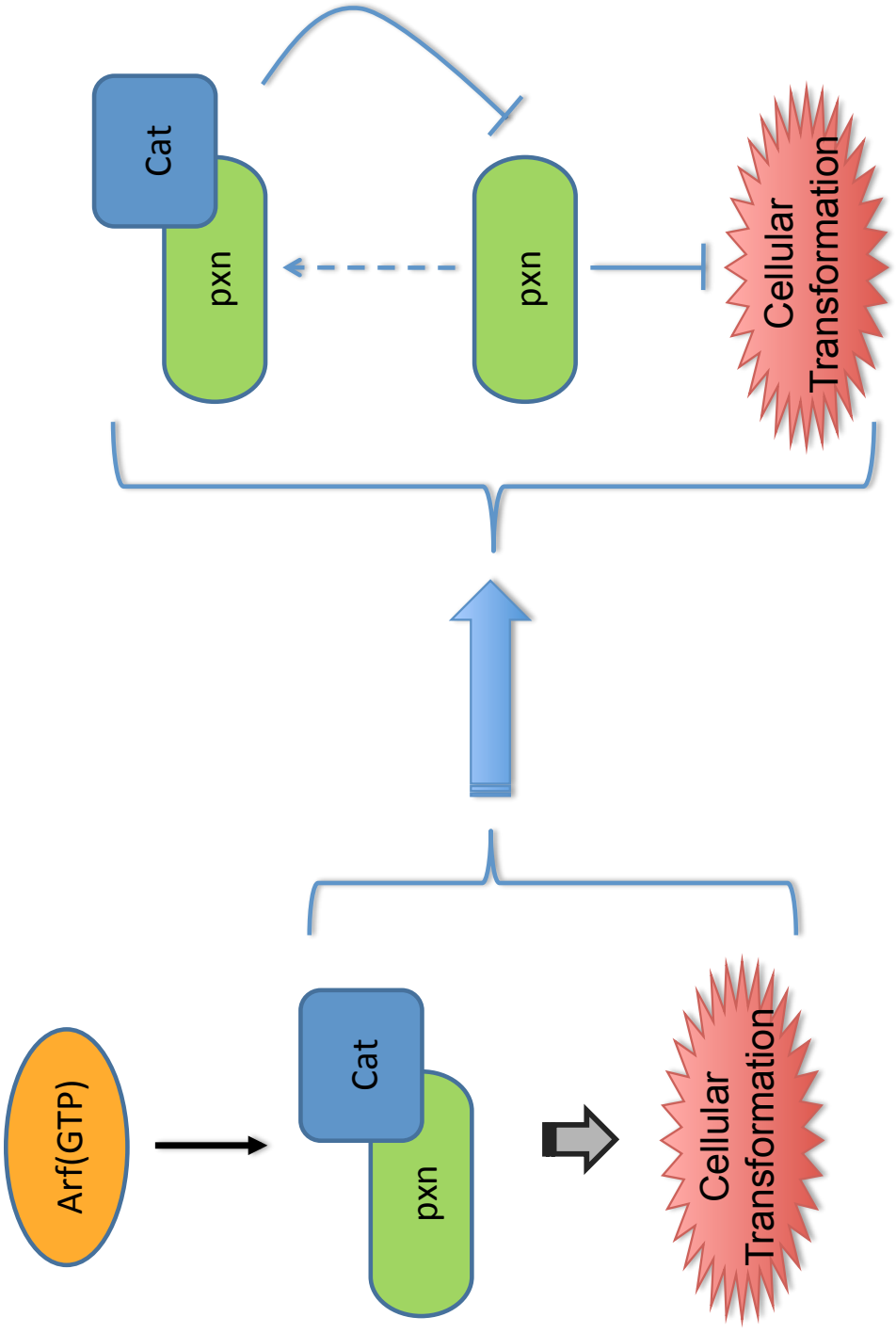
In chapter 2, I address this question and the findings highlight a novel role for Cat in promoting aberrant growth phenotypes of transformed cell lines and human cancer cell lines. In particular, the results show that Cat is necessary for Cdc42 F28L–mediated transformation as read-out by colony formation in soft agar. Also, Cat was frequently expressed at high levels in various human cancer cell lines. Since HeLa cervical carcinoma cells had one of the highest levels of Cat expression, I subjected a tissue micro-array composed of primary cervical carcinoma samples to immunohistochemical analysis using a Cat-1 antibody. The results from this experiment showed that over 95% of the cervical cancers had higher Cat expression than its normal tissue counterparts. I then went on to show that knocking-down Cat expression in HeLa cells blocked the anchorage-independent growth of the cells, confirming for the first time a role for Cat in promoting aberrant cell growth. These findings raise a number of important questions for further study. For example, what other types of cancer require Cat for their progression? Since pancreatic cancer cell lines and brain tumor cell lines also express Cat to fairly high levels, it would be interesting to probe for Cat expression in pancreatic and brain tumor tissue arrays. This will allow us to determine whether Cat over-expression is a feature unique to cervical cancers, or whether Cat may be deregulated in several different types of human cancers.

We then wanted to learn more about the mechanism underlying the Cat-mediated anchorage-independent growth of HeLa cells. The approach that we took to answer this question was to introduce siRNA-insensitive forms of Cat that were defective for a

specific function(s) into cells in which the endogenous Cat expression was knocked down by RNAi and see whether the block in cellular transformation could be restored, or 'rescued,' by a particular mutant form of Cat. This line of study led me to determine that the paxillin-binding defective form of Cat could not rescue the Cat knock-down phenotype, whereas an Arf GAP-defective Cat, led to an even greater extent of anchorage-independent growth compared to HeLa cells expressing the siRNA-insensitive wild type Cat construct. This enhanced ability to form colonies in soft agar was also dependent on the binding of Cat to paxillin. These results suggested that Cat might be working as an effector for Arf GTPases, and that paxillin binding is an important step in carrying-out the effector function (Figure 4.1). Such a model would predict that Arf GTPases could sufficiently drive cellular transformation, and their transforming ability would be dependent on Cat binding to paxillin. Indeed, introduction of activated forms of Arf1 and Arf6 GTPases, the two substrates for the Arf GAP activity of Cat, was sufficient to induce anchorage-independent growth in NIH3T3 cells. In addition, co-expressing an activated form of Arf6 with Arf GAP-defective Cat mutant, strongly induced colony formation in soft agar, supporting the idea that Arf GTPases can promote transformation through Cat. To the best of my knowledge, this is the first demonstration that Arf GTPases are sufficient to induce anchorage-independent growth. Moreover, these findings also show that Cat is an important mediator of Arf-driven cellular transformation.

Since the binding of Cat to paxillin is crucial for Cat's ability to promote cell growth, it was logical to then ask what role paxillin has in cellular transformation. Thus, I

**Figure 4.1 The molecular mechanism underlying the cellular transformation promoted by Cat.** Cat promotes transformation presumably by acting as an effector for activated Arf GTPases. A critical function of Cat as an effector is the binding of Cat to paxillin. Experimental results suggest that the binding of Cat to paxillin is important because Cat can inhibit the negative impact that paxillin has on cellular transformation through binding to paxillin.


Cat
pxn
pxn
Cellular Transformation
Arf(GTP)
Cat
pxn
Cellular Transformation

knocked-down paxillin in HeLa cells and, surprisingly, this led to the enhanced growth of colonies in soft agar. Such a result was unexpected, given the previous reports suggesting paxillin functions as an important mediator of the transformed phenotypes of several colon cancer cell lines and transformed fibroblasts (5,6). Also, ectopically expressing paxillin in HeLa cells led to a corresponding decrease in cell growth, suggesting that paxillin was limiting cell growth. This finding suggested that the binding of Cat to paxillin is important because Cat is preventing paxillin from negatively impacting transformation. With this model in mind, I hypothesized that if the binding of Cat to paxillin is the only necessary function of Cat to promote cellular transformation, then knocking-down paxillin together with Cat in HeLa cells might be sufficient to rescue the inhibition on anchorage-independent growth that accompanies knocking-down Cat alone. Indeed this was what we observed in HeLa cells, supporting the model where paxillin has a negative role in cellular transformation and that the up-regulation of Cat in transformed and cancer cell lines further counteract their effects (Figure 4.1).

Cell signaling events influenced by Cat were somewhat counter-intuitive in that the activation of many of the mitogenic signaling pathways were up-regulated under conditions where Cat expression was inhibited by siRNA. For example, knocking-down Cat expression led to increases in phosphorylated EGF receptor, Erk, Fak, and S6K activity. This suggested to me a couple of scenarios to explain the apparent discrepancy between the signaling events regulated by Cat and the results from soft agar assays where Cat was shown to promote oncogenic growth. One possibility is that a mitogenic signaling pathway that is critical for HeLa cell growth is down-regulated when Cat

expression is knocked-down. In fact, knocking-down Cat leads to a decrease in Akt phosphorylation, a key signaling protein that promotes survival, which may account for the inhibition of colony formation upon knocking-down Cat expression. Another possibility is that it is the up-regulation of the activation of signaling proteins that may be responsible for inhibiting cellular transformation when Cat is knocked-down in HeLa cells. Such idea is supported by the fact that I was able to partially rescue the block in cell growth caused by introducing Cat siRNA into HeLa cells by treatment with rapamycin, an inhibitor of mTOR kinase, which blocks the mTOR / S6Kinase pathway (results not shown). Excessive mTOR-signaling that accompanies the knock-down of Cat RNAi in HeLa cells, may be driving cells to apoptosis. In fact, there have been several studies in the literature that showed similar results where treatment of rapamycin led to the promotion of cell growth (7-10). One commons feature of these reports was the absence of the Lkb signaling pathway in the cell systems that were being used in these studies (7-10). Namely, in cells where the Lkb kinase, or any of its downstream effectors such as the AMP Kinase or Tumor Sclerosis Complex proteins were knocked down, treatment of these cells with rapamycin led to enhanced cell growth. Lkb kinase, and its downstream effector the AMP Kinase have a major role in detecting low ATP levels in cells. They are activated by high levels of AMP or ADP in order to down-regulate mTOR signaling when there is little energy available in cells. The Lkb-signaling pathway that down-regulates cell growth by inhibiting mTOR activity in effect acts to keep cells from undergoing energy failure-induced apoptosis. Indeed HeLa cells do not express the Lkb kinase supporting this hypothesis. Thus, it will be interesting to see whether Cat is able to tune-down excessive signaling by the mTOR pathway.

One obvious candidate protein that acts downstream of Cat and can influence mitogenic signaling is, again, paxillin. In agreement with the idea that paxillin acts downstream of Cat, and the observation that knocking-down Cat leads to activation of mitogenic signaling pathways, the over-expression of paxillin alone can promote mitogenic signaling in cells (results not shown, (6,11)). Moreover, upon knocking-down paxillin expression acts to deactivate the signaling pathways that are activated upon knocking-down Cat. Also, in agreement with the idea that paxillin is a negative regulator of anchorage-independent growth, over-expressing paxillin in HeLa cells led to decreased number of colonies formed in soft agar, despite activating many signaling pathways. Importantly, however, over-expressing the paxillin Y-F (Tyr31Phe, Tyr118Phe) mutant, did not have any effect on the anchorage-independent growth of HeLa cells. This suggested that the two tyrosines on paxillin were mainly responsible for mediating the negative effects of paxillin on the anchorage-independent growth of HeLa cells. What protein could be mediating such role? One of the best known signaling proteins that interacts with the two tyrosines on paxillin is Crk. Crk is an adapter protein with one SH2 and two SH3 domains, and despite not having a catalytic domain, expression of v-Crk in cells induces a large number of tyrosine-phosphorylated signaling proteins and is strongly transforming (12). It is tempting to think of a putative model where Cat, by binding to paxillin, is preventing paxillin from recruiting Crk to sites where Crk mediates the activation of signaling molecules. Perhaps, by binding to paxillin, Cat might be affecting paxillin's tyrosine phosphorylation that is necessary for the recruitment of Crk. Since Crk has been shown to have such prominent roles in both receptor tyrosine kinase signaling

and also integrin signaling, this model might explain how knocking-down Cat can lead to hyper-phosphorylation of many signaling proteins. Crk is also an attractive candidate for specifically mediating the negative impact of paxillin on transformation because Crk does not bind Hic-5, in accordance with my data that suggests it is paxillin but not Hic-5 that has a critical role for Cat-mediated transformation.

What is the general picture that emerges from these findings? It seems that Cat is an ArfGAP and also an effector of Arf GTPase that, by coordinating these two roles, functions in regulating mitogenic signaling potentially by affecting lipid trafficking through binding to paxillin. Important questions that lie ahead are as follows;

1) What is the effector function of Cat that promotes anchorage independent growth? How does Cat cooperate with the activated Arf6 and Arf1 to promote such function?

2) Does Cat work in general to down-regulate mitogenic signaling, and if so, how does Cat do it? Is this at the level of the receptor tyrosine kinase/integrin endocytosis or recycling?

3) Why does knocking-down Cat result in activation of mitogenic signaling when, at the same time block anchorage-independent growth? Since this happens in both HeLa cells and NIH3T3 cells that express the oncogenic Cdc42 F28L mutant, what is common about these cell lines?

4) How do Cdc42 and Arf6/Arf1 coordinate their actions to carry out cellular functions through Cat?

5) How is Cat's GAP function activated? Is the binding of Cat to paxillin a necessary step?

Cat is a novel protein to be found to have a role in tumorigenesis. This protein is especially interesting in that Cat immediately implicates Arf GTPase in cancer as well. A good deal has been already studied about how Cdc42/Rac and Arf GTPase affect cell morphology and directional migration. Understanding how these GTPases work together with Cat to affect aberrant growth will be interesting and could also have broad implications.

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